# The Role of Galectin-1 on Neutrophil Recruitment and Clearance in the Arthritic Joint

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## Declaration

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## Abstract

Galectins are a family of endogenous proteins defined by their binding specificity for β-galactosides. Galectin-1 (Gal-1) exerts immune-regulatory and anti-inflammatory actions. Its release into the extracellular milieu often correlates with the peak of inflammation suggesting that it is induced during inflammation and may serve a pro-resolving function. Indeed, Gal-1 has been shown to be protective in T cell driven models of autoimmunity largely through its ability to induce T cell apoptosis, yet its effects on innate immune cells such as neutrophils remains to be fully elucidated.

The persistent recruitment of neutrophils to the arthritic joint and their prolonged survival within synovial fluid is a major factor in the pathogenesis of rheumatoid arthritis and thus an attractive therapeutic target. This thesis presents research on the role of Gal-1 in neutrophil trafficking, apoptosis, and clearance in an animal model of inflammatory arthritis, supported by a model of acute self-resolving peritonitis and *in vitro* assays.

In response to arthritogenic serum Gal-1 KO mice exhibited delayed disease progression compared to WT mice as well as significantly reduced paw oedema and weight loss, however both genotypes reached a comparable clinical score at the peak of disease. Importantly, remission of arthritis was delayed in Gal-1 KO mice, with a resolution interval of 16 days versus 12 days in WT counterparts, suggesting a defect in the clearance of inflammatory cells.

To investigate this further, a model of acute self-resolving inflammation was used to profile cell recruitment and clearance. Gal-1 KO mice displayed enhanced neutrophil recruitment compared to WT. Furthermore, the administration of recombinant Gal-1 (hrGal-1) following the peak of inflammation led to reduced neutrophil numbers at 24 and 48 hours, shortening the resolution interval from 39 to 14 hours.

In line with the hypothesis that Gal-1 enhances clearance in an inflammatory setting, assessment of neutrophil apoptosis *in vitro* demonstrated that although Gal-1 had little effect on survival when given alone it significantly reversed the pro-survival signals of GM-CSF, increasing neutrophil apoptosis.

In conclusion, the delayed remission of arthritis observed in Gal-1 KO mice coupled with the ability of hrGal-1 to enhance resolution of acute inflammation and counter-regulate neutrophil pro-survival signals is suggestive of a role for Gal-1 in neutrophil clearance and thus the timely resolution of acute inflammation.

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| | |
|---|---|
| ΔΨm | Mitochondrial membrane potential |
| AA | Arachidonic acid |
| ACPA | Anti-citrullinated protein antibody |
| AIA | Adjuvant induced arthritis |
| AnxA1 | Annexin A1 |
| AnxV | AnnexinV |
| APC | Antigen-presenting cell |
| ARE | AU-rich element |
| BAI1 | Brain-specific angiogenesis inhibitor 1 |
| BMDM | Bone marrow derived macrophages |
| BMI | Body mass index |
| bp | Base pair |
| BSA | Bovine serum albumin |
| CII | Collagen type-II |
| CAIA | Collagen antibody induced arthritis |
| Caspases | Cysteine-aspartic proteases |
| CCL | C-C motif ligand |
| CCR | Chemokine receptor |
| CD | Cluster of differentiation |
| CFSE | Carboxyfluorescein Succinimidyl Ester |
| CIA | Collagen-induced arthritis |
| CID | Chronic inflammatory diseases |
| COX | Cyclooxygenase |
| CR | Complement receptor |

| | |
|---|---|
| CRD | Carbohydrate-recognition domain |
| CTLA4 | Cytotoxic T-lymphocyte associated protein 4 |
| CV | Cardiovascular |
| CXCL | C-X-C motif ligand |
| DAMP | Damage associated molecular pattern |
| $DiOC_6$ | 3,3′-dihexyloxacarbocyanine iodide |
| DMARD | Disease-modifying anti-rheumatic drug |
| DMSO | Dimethyl Sulfoxide |
| DNA | Deoxyribonucleic acid |
| DNAse | Deoxyribonuclease |
| DPBS | Dulbecco’s phosphate buffered saline |
| DTT | 1,4-Dithiothreitol |
| EAE | Experimental Autoimmune encephalomyelitis |
| EAMG | Experimental autoimmune models of myasthenia gravis |
| ECM | Extracellular matrix |
| *E. coli* | *Escherichia coli* |
| EDTA | Ethylenediaminetetraacetic acid |
| EndoG | Endonuclease G |
| ESAM | endothelial cell-selective adhesion molecule |
| ERK | Extracellular signal regulated kinase |
| FACS | Fluorescent activated cell sorting |
| Fas-L | Fas ligand |
| FBS | Foetal Bovine Serum |
| Fc | Fragment crystallisable region |
| FcγR | Fragment crystallisable region gamma receptor |

| | |
|---|---|
| FcR | Fragment crystallisable region receptor |
| FLS | Fibroblast like synoviocytes |
| fMLP | N-formyl-Methionyl-Leucyl-Phenylalanine |
| FSC | Forward light scatter |
| FSC-A | Forward scatter area |
| FSC-H | Forward scatter height |
| G6PI | Glucose-6-phosphate isomerase |
| Gal | Galectin |
| Gas6 | Growth arrest-specific 6 |
| G-CSF | Granulocyte colony-stimulating factor |
| GM-CSF | Granulocyte macrophage colony-stimulating factor |
| H&E | Haematoxylin and Eosin |
| $H_2O_2$ | Hydrogen peroxide |
| HLA | Human leukocyte antigen |
| HOCl | Hypochlorous acid |
| hr | Human recombinant |
| HRP | Horseradish peroxidase |
| HTLV-1 | Human T lymphotropic virus type-1 |
| HUVEC | Human umbilical vein endothelial cells |
| IA | Inflammatory arthritis |
| IC | Immune complex |
| ICAM | Ig superfamily of cell adhesion molecules |
| IFA | Incomplete Freund’s adjuvant |
| IFN-γ | Interferon-gamma |
| IgG | Immunoglobulin |

| | |
|---|---|
| IL | Interleukin |
| Inos | Inducible nitric oxide synthase |
| i.p. | Intra-peritoneal |
| IVC | Individually ventilated cage |
| JAK | Janus kinase |
| KO | Knockout |
| L-glut | L-glutamine |
| LDS | Lithium dodecyl sulphate |
| LFA | Lymphocyte function-associated antigen |
| LOX | Lectin-like oxidized low-density lipoprotein receptor |
| LPS | Lipopolysaccharide |
| LRSC | Leukocyte reduction system chamber |
| $LTB_4$ | Leukotriene B4 |
| LXR | Liver x receptor |
| Mac-1 | Macrophage-1 antigen |
| MAPK | Mitogen-activated protein kinase |
| MBL | Mannose binding lectin |
| MFG-E8 | Milk fat globule EGF factor 8 |
| MFI | Median fluorescence intensity |
| MHC | Major histocompatibility complex |
| MIF | Macrophage inhibitory factor |
| MK2 | MAPK-activated protein kinase 2 |
| MMP | Matrix metalloproteinase |
| MRL | Murphy Roths Large |
| NADPH | Nicotinamide adenine dinucleotide phosphate |

| | |
|---|---|
| NBF | Neutral buffered formalin |
| NET | Neutrophil extracellular trap |
| NIR | Near infrared |
| NO | Nitric oxide |
| NOD | Non-obese diabetic |
| $O_2^-$ | Superoxide |
| OxPS | Oxidised phosphatidylserine |
| P/S | Penicillin-Streptomycin |
| PAD-4 | Peptidyl arginine deiminase-4 |
| PAF | Platelet activating factor |
| PAMP | Pathogen-associated molecular pattern |
| PBMC | Peripheral blood mononuclear cell |
| PC | Phosphatidylcholine |
| PCR | Polymerase chain reaction |
| PE | Phosphatidylethanolamine |
| PECAM | Platelet/endothelial cell adhesion molecule 1 |
| PFA | Paraformaldehyde |
| $PGE_2$ | Prostaglandin E2 |
| PGES | Prostaglandin E synthase |
| $PGI_2$ | Prostacyclin |
| PI | Propidium iodide |
| PI3-kinase | Phosphoinositide 3-kinase |
| Poly-LacNAc | Poly-*N*-acetyllactosame |
| PPAR | Peroxisome proliferator-activated receptor |
| ProS1 | Protein S |

| | |
|---|---|
| PS | Phosphatidylserine |
| PSR | Phosphatidylserine receptor |
| PSGL-1 | P-selectin glycoprotein ligand 1 |
| PTPN22 | Protein tyrosine phosphatase, non-receptor type 22 |
| RA | Rheumatoid arthritis |
| RAGE | Receptor for advanced glycosylation end products |
| RAMP | Resolution associated molecular pattern |
| RANK | Receptor activator of nuclear factor-κB |
| RANKL | Receptor activator of nuclear factor-κB ligand |
| RBC | Red blood cell |
| RF | Rheumatoid factor |
| Ri | Resolution interval |
| RNase | Ribonuclease |
| ROS | Reactive oxygen species |
| RPMI | Roswell Park Memorial Institute |
| RT | Room temperature |
| SAA | Serum amyloid A |
| SKG | Sakaguchi |
| SSC | Side light scatter |
| SSC-A | Side scatter area |
| SSC-W | Side scatter width |
| STAT4 | Signal transducer and activator of transcription 4 |
| STIA | Serum transfer induced arthritis |
| $T_{50}$ | 50% of the maximum magnitude of inflammatory response |
| TAE | Tris-acetate EDTA |

| | |
|---|---|
| TAM | Tyro3, Axl, and MerTK |
| TBS | Tris buffered saline |
| TBS-T | Tris buffered saline with Tween |
| TCR | T-cell receptor |
| Th | T helper |
| Tim | T cell immunoglobulin and mucin domain-containing molecule |
| $T_{max}$ | Maximum magnitude of inflammatory response |
| TLR | Toll-like receptor |
| TNF | Tumour necrosis factor |
| TRAIL | TNF-related apoptosis-inducing ligand |
| Twy | Tiptoe walking-Yoshimura |
| VCAM | Vascular cell adhesion molecule |
| WBC | White blood cell |
| w/v | Weight/volume |
| WT | Wildtype |
| ZAP-70 | Zeta-chain-associated protein kinase 70 |

# Chapter 1: Introduction

## *1.1. Inflammation and Resolution*

Inflammatory responses initially arise when the equilibrium of a usually constant or relatively stable environment is disturbed. Despite tight regulation by the immune system, an abnormal response means the return to homeostasis is not always restored. Instead, the mediators that initiate, maintain and shut down the inflammatory response become imbalanced (Choy, 2011) commonly resulting in the persistent infiltration of inflammatory cells.

Often considered the hallmark of an inflammatory response, the recruitment of leukocytes from the bloodstream to the surrounding tissue is a fundamental feature in both acute and chronic inflammation (Cooper *et al.*, 2012). Traversing the endothelial barrier first are neutrophils. Acting as the first responders, neutrophils are phagocytes that arrive at the site of inflammation with powerful enzymes, oxidants and antimicrobial properties (Hampton *et al.*, 1998). The migration of neutrophils out of the circulatory system towards the target tissue site initiates a stream of other mononuclear leukocytes to follow. Depending on the stimulus, T lymphocytes or monocytes are next to take up a role within the multistep process. In the case of acute inflammation, bioactive mediators are produced to promote resolution, bringing the short-term immune response to an end (Serhan and Savill, 2005).

To return the targeted site to homeostasis, cessation of infiltration and disposal of the already present cells is essential (as shown in summary figure 1). To facilitate the

latter, inflammatory leukocytes must take one of two main exit routes: cells that remain live and viable will most often re-enter the systemic circulatory system whilst cells that have undergone local cell death are targeted for efferocytosis. Regardless of the assigned course, cell clearance needs to be both controlled and effective (Serhan *et al.*, 2007), requiring a switch in the cellular environment from pro-inflammatory to pro-resolving. Evidence indicate that resolution of inflammation is an active biosynthetic programmed response regulated by the production of mediators with specialised actions (Fredman *et al.*, 2012).

If the resolution process fails, persistent recruitment and infiltration of leukocytes into the tissue may occur, resulting in chronic inflammation. Therefore although the swift recruitment of immune cells is required for effective host defence, such recruitment has a detrimental effect when directed to healthy peripheral tissues (Sadik and Luster, 2012), whereby either the unrequired recruitment of immune cells or retention of the inflammatory milieu facilitates the pathogenesis of autoimmune inflammatory diseases, such as Rheumatoid Arthritis (RA).

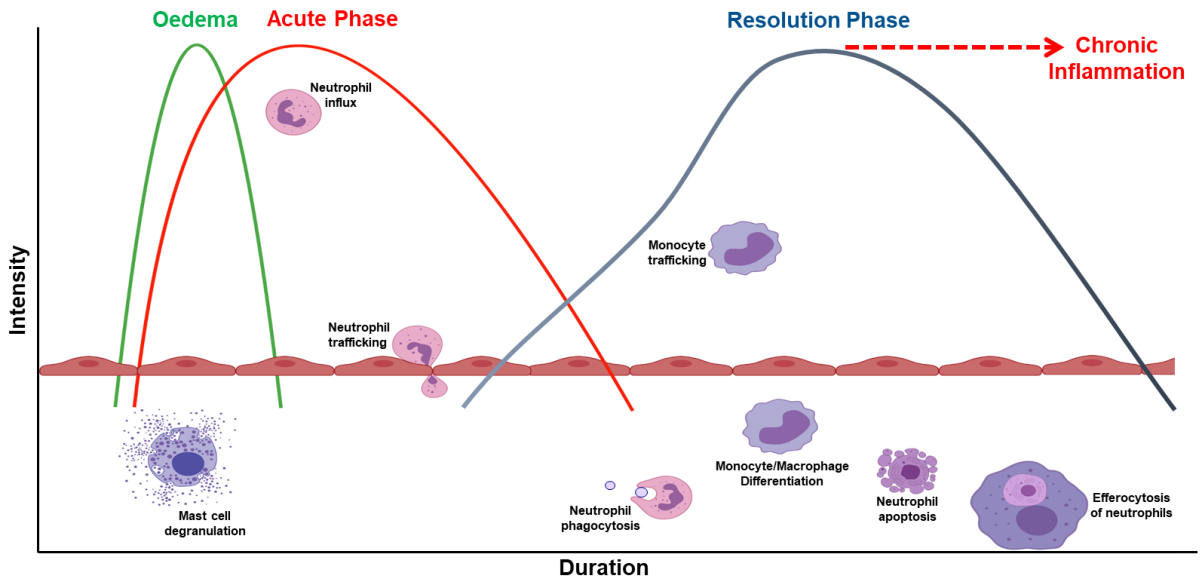



**Figure 1. Dynamics of inflammation and resolution.**
The inflammatory response occurs in a number of co-ordinated and temporal phases. Inflammatory mediators initiate an innate immune response characterised by rapid mast cell degranulation and mass infiltration of neutrophils, respectively resulting in oedema and the onset of an acute phase response. Recruited neutrophils are followed by monocytes that differentiate into macrophages in the tissue. Once migrated into the tissue, leukocytes produce numerous mediators, which govern the profile of the inflammatory response. The switch in mediator production from pro-inflammatory to pro-resolving is critical to the shift from inflammation to resolution. In the resolution phase, neutrophils, which have performed their role phagocytosing foreign material, undergo apoptosis and are subsequently cleared by macrophages termed efferocytosis. An imbalance of these behaviours can dysregulate the inflammatory response and prohibit resolution, in this case chronic inflammation ensues. [Created using BioRender illustrating tool.]

### *1.2. Neutrophils*

As the most abundant white blood cell in humans, neutrophils account for between 50-70% of leukocytes (compared with 10-25% in mice). Neutrophils undergo diapedesis to quickly migrate towards sites of inflammation and once positioned can provide an anti-microbial function through processes such as NETosis, phagocytosis and degranulation, thereby having a crucial role in clearance of infection. As the understanding of the inflammatory response has progressed neutrophils are now also regarded for their facilitation in chronic inflammatory conditions and adaptive immune responses (Caielli *et al.*, 2012; Soehnlein *et al.*, 2017).

Under physiological conditions the bone marrow, spleen, liver and lungs serve as reservoirs of mature neutrophils (Summers *et al.*, 2010). In particular, a large number of mature pulmonary neutrophils exist (Sibille and Marchandise, 1993; Kolaczkowska and Kubes, 2013), persisting for longer than commonly seen at other sites (Ussov *et al.*, 1995). It is generally considered that the concentrated populations in the aforementioned organs provide two main functions, both constantly patrolling their environment for any disturbances as well as being braced for cues to swiftly migrate in response to signals of disruption.

Circulating neutrophils are short lived. Cells maintained in the periphery have a half-life of only 8 hours (Galli *et al.*, 2011) and although neutrophils that have traversed the endothelium in to the tissue are afforded a more extended lifespan, under regulatory conditions they will still die after 1-2 days (Blumenreich, 1990). However at sites of inflammation the presence of entities that induce pro-survival signalling (including certain cytokines (A. S. Cowburn *et al.*, 2002), bacterial products (Lee *et al.*, 1993)

and a hypoxic environment (Hannah *et al.*, 1995)) extend neutrophil longevity resulting in sustained presence of primed neutrophils. In any case, with a continuous demand for fresh neutrophils promyelocytic progenitors in the bone marrow are tasked with generating approximately $10^{11}$ new neutrophils daily (Rosales, 2018).

### *1.2.1. Neutrophil Trafficking*

Neutrophil recruitment from the vasculature to the tissue occurs in several sequential steps, commonly recognised to be tethering, rolling, adhesion, crawling and transmigration, as summarised in figure 2. Inflammatory mediators initiate changes on the surface of the endothelium such that expression of adhesion molecules (P-selectin and E-selectin) are increased. Specifically, pre-stored P-selectin is upregulated and facilitates the tethering/capturing of free circulating neutrophils, with a partially overlapping function, E-selectin is synthesized *de novo* and later expressed (Bevilacqua *et al.*, 1989; Hattori *et al.*, 1989; Geng *et al.*, 1990). Neutrophils continue to form ‘catch slip’ bonds with these ligands thus ‘rolling’ along the endothelium (Sundd *et al.*, 2011).

Upon activation, the surface of the endothelium becomes adorned with chemokines. Upregulation of the ELR-CXC chemokines, which signal via CXCR2, is particularly significant for neutrophil adhesion to the endothelium (Girbl *et al.*, 2018). For firm adhesion to take place, binding of integrins (transmembrane receptors) to cell adhesion molecules (on the endothelial cell surface) is essential. Binding of integrins to their respective cell-surface ligands triggers internal neutrophil pathways and as such is assigned the term ‘outside-in signalling’ which acts to steady adhesion and

start cell motility (Fan and Ley, 2015). At this stage neutrophils are prepared for transmigration.

Junctions formed between neighbouring endothelial cells serve as the preferred sites for transmigration and neutrophils will actively ‘crawl’ towards these spots (Phillipson *et al.*, 2006; Shulman *et al.*, 2009). The elongated endothelial cell architecture that has resulted from shear stress, aids this integrin-mediated behaviour whereby neutrophils can move with, against or perpendicular to the direction of blood flow as required (Sumagin *et al.*, 2010).

The process of transmigration out of the vasculature occurs in two phases, the cell must first pass through the endothelium before then crossing the basement membrane. In both cases interactions between integrins and members of the Ig superfamily of cell adhesion molecules (ICAMs), including ICAM-1 (CD54) and ICAM-2 (CD102), have a key role. Other essential molecules include vascular cell adhesion molecule 1 (VCAM-1/CD106) as well as junctional proteins including; platelet/endothelial cell adhesion molecule 1 (PECAM-1/CD31), junctional adhesion molecules (JAMs), endothelial cell-selective adhesion molecule (ESAM) and the cell surface glycoprotein CD99 (Wegmann *et al.*, 2006; Woodfin *et al.*, 2009; Voisin and Nourshargh, 2013; Muller, 2016).

Once in the tissue emigrated neutrophils must continue to navigate towards their destination. It is suggested that this occurs due to a hierarchy of chemotactic molecules (in infected tissues in comparison to the endothelium) with the ability to dethrone previous stimuli. In this manner neutrophils advance from following

'intermediate' to favouring 'end-target' chemoattractants, with the 'go' and 'stop' instructions shown to be signalled via mitogen-activated protein kinase (MAPK) pathways, specifically p38 MAPK and extracellular signal regulated kinase (ERK) (Liu *et al.*, 2015).

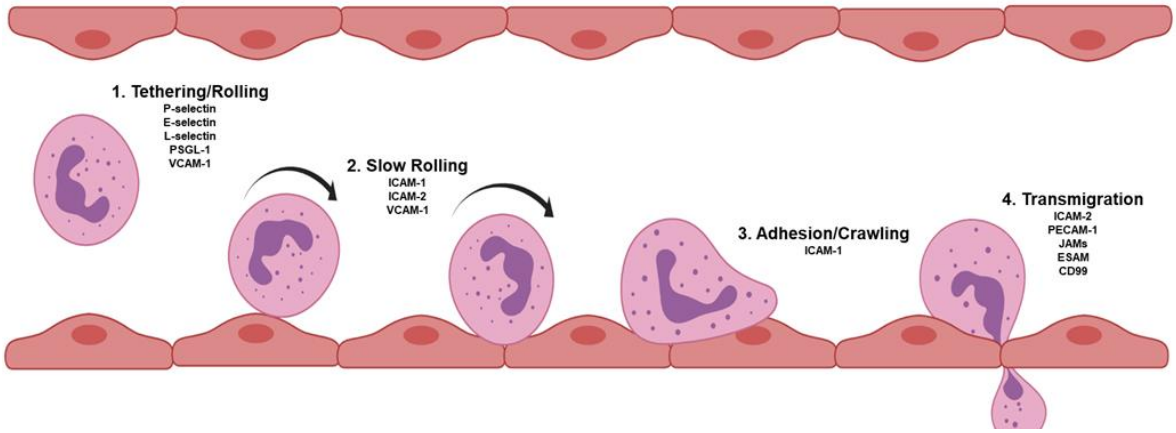



**Figure 2. Neutrophil trafficking.**
Upon stimulation tissue resident monocytes/macrophages release inflammatory mediators, which signal for neutrophil recruitment from the vasculature to the tissue. A sequential process of tethering, rolling, adhesion, crawling and finally transmigration facilitate neutrophil trafficking along the endothelium. (**1**) Inflammatory mediators induce rapid expression of adhesion molecules (pre-formed P-selectin and newly synthesised E-selectin) on the surface of the endothelium. (**2**) Selectins form 'catch slip' bonds with their glycoprotein ligands on the neutrophil surface promoting a 'rolling' motion along the endothelium. (**3**) Chemokines on the endothelium activate integrins on the surface of neutrophils, which bind firmly to cell adhesion molecules on the endothelium and neutrophils actively 'crawl' towards migratory junctions between neighbouring endothelial cells. (**4**) Interactions between integrins and adhesion molecules support neutrophil migration, first through the endothelium and then traversing the basement membrane, into the tissue. [Created using BioRender illustrating tool.]

### *1.2.2. Neutrophil Functions*

Evidence is beginning to emerge that indicates the existence of different neutrophil subtypes. Their pleiotropic functions under pathologic conditions, but also at steady state, suggests that various neutrophil populations exist. This heterogeneity provides for diverse immunologic roles (including phagocytosis, degranulation, respiratory burst, NETosis, microparticle release and cytokine production) and selective recruitment from the circulation (Christoffersson *et al.*, 2012). Furthermore neutrophil production of cytokines and chemokines and membrane expression of Toll-like receptors (TLRs) and integrins has been shown to exist in diverse arrangements (reviewed in (Rosales, 2018)). As such, these variable characteristics determine neutrophil phenotype (pro-inflammatory or anti-inflammatory) and consequently the course of the immunological response and induction of macrophage phenotype (M1 or M2 respectively) (Tsuda *et al.*, 2004; Jaiswal *et al.*, 2009; Pillay *et al.*, 2012; Chen *et al.*, 2014).

In general, neutrophil behaviour is a direct response to stimuli, either from the surrounding environment or from intrinsic cues. These external and internal factors, such as recognition of pathogens or survival signals respectively, will dictate their actions. Neutrophils can then directly and/or indirectly shape both the innate and adaptive immune responses. The importance of neutrophil anti-inflammatory and/or pro-resolving actions has long been established for acute immune responses however emerging research suggests the potential of these behaviours for taming chronic inflammation (as discussed in (Jones *et al.*, 2016)). Indeed, production of neutrophil extracellular-traps (NETs) and microvesicles, have been shown within a variety of inflammatory microenvironments to aid in combating inflammation (Schauer *et al.*,

2014; Rhys *et al.*, 2018). Exuberant immune cell activation resulting in over production of inflammatory mediators can be dampened by neutrophil driven mechanisms with some neutrophil phenotypes suggested to contribute to the resolution of inflammation through their release of anti-inflammatory mediators (Cuartero *et al.*, 2013). Furthermore, neutrophils are the major phagocyte of innate immunity and their ability to rapidly clear invading entities is an essential role in host defence (van Kesse *et al.*, 2014).

Degranulation is the main mechanism utilised by neutrophils for the release of their mediators contained in granules. The act of degranulation may occur in one of two distinct ways. For smaller granules, that are more easily stimulated and mobilised, internal vesicles fuse with the plasma membrane and their content spills out of the cell. Alternatively, a phagolysosome is formed by internal vesicles binding with phagocytic vacuoles and delivering antibacterial proteins and enzymes (Nauseef and Borregaard, 2014; Cowland and Borregaard, 2016). In any case the substrates found contained in granules are potentially toxic meaning that it is critical that they are well contained and tightly regulated in order to avoid damage both intrinsically (neutrophil auto destruction) and extrinsically (inflammation and host tissue damage). It is universally accepted that excessive neutrophil degranulation is a common feature of many inflammatory disorders (Lacy, 2006).

The respiratory burst, or oxidative burst, is the rapid release of reactive oxygen species (ROS). The toxic metabolites including nitric oxide (NO), superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$) as well as cytochrome b558 (the central component of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase), are generated as

additional contents within neutrophil granules (Nguyen *et al.*, 2017). Respiratory burst plays a particularly valuable role in the immune system, with NADPH oxidase crucial in the degradation mechanisms following phagocytosis. The production of oxidants is commonly considered to be the most powerful defence mechanism utilised by the neutrophil. However, under certain circumstances such as during RA, neutrophils can be triggered to release ROS and proteases extracellularly causing damage to host tissues, worsening disease (Glennon-Alty *et al.*, 2018).

Neutrophils have also been shown to have a role in the production of pro-inflammatory mediators, with potent examples including arachidonic acid (AA) metabolites and certain cytokines. Particularly during acute inflammation neutrophils enzymatically biosynthesise leukotrienes and prostaglandins from AA. Leukotrienes are synthesised by inflammatory cells via the 5-lipoxygenase (5-LO) pathway, with neutrophils specifically producing leukotriene B4 ($LTB_4$). $LTB_4$ is a particularly potent mediator of chemoattraction and can induce cell adhesion and activation (Samuelsson *et al.*, 1987). Presence of the enzymes cyclooxygenase (COX) and terminal prostaglandin E synthases (PGES) results in AA being transformed into prostaglandin $E_2$ ($PGE_2$) (Park *et al.*, 2006), an inflammatory mediator possessing potent effects.

Numerous cytokines/chemokines are produced by neutrophils (e.g. IL-8, IL-12, TGF-β and MCP-1, to name a few). Although the amount of cytokine produced is relatively small in comparison to other leukocytes the number of neutrophils present at inflammatory sites is much larger, thus neutrophil cytokine production has the potential to contribute to inflammation (Cassatella, 1999).

A number of neutrophil derived anti-inflammatory and pro-resolving products also exist, including Annexin A1 (AnxA1) and lipid mediators (e.g. resolvins and lipoxins). Neutrophils contain AnxA1 within their tertiary (gelatinase) granules and cytosol (Perretti and Flower, 2004). In response to high intracellular $Ca^{2+}$ AnxA1 will localise to the plasma membrane to bind phosphatidylserine. Furthermore, direct neutrophil and AnxA1 interaction functions to inhibit recruitment to sites of inflammation as well as to induce neutrophil apoptosis, thus promoting efferocytosis by macrophages. Mechanistically the latter occurs as a consequence of AnxA1 attracting recruitment of monocytes and macrophages following its secretion from neutrophils as they undergo apoptosis (Jones *et al.*, 2016). Promotion of neutrophil apoptosis and clearance in this manner is essential to the resolution of both acute and chronic inflammation.

Lipoxins and resolvins can also promote neutrophil apoptosis. The uptake of apoptotic neutrophils by resident macrophages promotes lipoxin A4 production, resulting in lipid class switching from the 5-LO pathway (leukotriene and prostaglandin synthesis) to the 15-LO pathway (Freire-de-Lima *et al.*, 2006). Resolvins act to reduce vascular permeability (consequently reducing neutrophil recruitment) and induce neutrophil apoptosis (subsequently increasing monocyte/macrophage recruitment and efferocytosis). Specifically, D-series and E-series resolvins mediate increased chemokine receptor five (CCR5) expression on apoptotic neutrophils thus providing them with the ability to ‘scavenge’ (gather and reduce) chemokines (e.g CCL3 and CCL5) (Ariel *et al.*, 2006). A decrease in further cell recruitment and activation occurs as a result of the lower concentration of these soluble chemokines.

Identification of neutrophils extracellular traps (NETs) is a comparatively recent finding within neutrophil biology, nonetheless rapid advances have been made in many areas of their study including structure/composition, anti-microbial activity and regulation of NETosis. Alongside phagocytosis and secretion of mediators, NETs are principally considered a novel third mechanism for killing invading pathogens, with their release providing neutrophils a final act of killing at the end of their lifespan (Fuchs *et al.*, 2007). NETs are networks of extracellular meshes composed mainly of chromatin with neutrophil granular proteins attached (Brinkmann *et al.*, 2004). Upon cellular activation NETs are released outside the cell to be formed in tissues and within blood vessels as a form of innate response. These extracellular fibres bind microorganisms, preventing their spreading. Aggregation of the fibres forms larger threads, generally around 50nm in diameter (Brinkmann *et al.*, 2004) however larger structures have been detected under flow conditions (Clark *et al.*, 2007). Their scaffold-like structure simultaneously provides a physical barrier and facilitates trapping of bacteria whilst also locally concentrating anti-microbial components. These high localised concentrations consequently bind, disarm and kill microbes thus providing an extracellular mechanism of killing independent of phagocytic uptake.

One major caveat to the process of NETosis is that more often than not it will result in the death of the neutrophil. Therefore, it is commonly referred to as ‘suicidal NETosis’ as a result of the processes of chromatin decondensation, nuclear swelling and membrane perforation that occur. However, it is worth noting that NETosis does not universally result in destruction of the neutrophil. Although occurrence is less common ‘vital NETosis’ also takes place, in this case nuclear material is still lost but instead of rapid formation and release blebbing of the nucleus occurs. As a consequence of the

mechanistic differences the neutrophil maintains its ability to chemotax and phagocytose (Yipp and Kubes, 2013). Following neutrophil activation the regulation of NET formation is primarily controlled by the lipoxygenase pathway (Clark *et al.*, 2011). Afterwards clearance of the released content is facilitated by macrophages in a manner similar to the clear up of apoptotic neutrophils (Farrera and Fadeel, 2013) .

Phagocytosis is utilised by the immune system for the removal of foreign entities, the process involves engulfment, digestion and expulsion of the waste products. Although one of the main functions of neutrophils, it is not unique to this cell type and several other types of white blood cells including monocytes (Dale *et al.*, 2008), macrophages (Silva and Correia-Neves, 2012) and dendritic cells also function as phagocytes (Savina and Amigorena, 2007). The difference in small molecule motifs on the surface of harmful particles, such as bacteria and viruses, from those on indigenous cells, enables detection of an infectious agent and alerts the immune response. Human neutrophils express Toll-like receptors (TLRs), which recognize the structurally conserved molecules, specifically known as pathogen-associated molecular patterns (PAMPs), as unusual. This antigen recognition step is aided by the process of opsonization, whereby the attachment of opsonins is especially important for engulfment by neutrophils, which are generally poor at phagocytosing unmodified targets (Shah *et al.*, 2017).

The efficiency of neutrophil phagocytosis has important implications in inflammation due to the ability of these cells to express varying Fc receptors (FcR). Under steady state neutrophil surface receptors detect and interact with the Fc portion of complexed or aggregated IgG, however upon stimulation (e.g. by infectious agents, interferon or

G-CSF) neutrophils will express receptors that recognise monomeric IgG. In addition to receptor switching, polymorphisms in the amino acids coding these receptors can also reduce phagocytic efficiency (Shah *et al.*, 2017). Ineffective FcR recognition of complexed IgG has significant impact in inflammatory diseases, such as Rheumatoid Arthritis, in which immune complexes (ICs) have a facilitating role. Furthermore, recent studies have shown that neutrophils engaged in FcγR-dependent IC phagocytosis undergo accelerated apoptosis (Gamberale *et al.*, 1998).

A very specific form of phagocytosis is efferocytosis, where phagocytes clear dead cells that have undergone apoptosis. Clearance of apoptotic neutrophils by macrophages in this manner has a particularly important role in inflammation (as described in section 1.2.4).

### *1.2.3. Neutrophil Apoptosis*

Although a choreographed response, the increased neutrophil number and activity at sites of inflammation has the capacity to have adverse effects if the cell population prevails. Fortunately, following their activity, neutrophils are inherently programmed to undergo cell death via apoptosis. This initiation of self-destruction is both normal and controlled. Apoptosis is crucial for the maintenance of tissue homeostasis in both the short and long term. Not only does it prevent the extracellular release of the neutrophil's cytotoxic contents, conferring short-term protection, it also facilitates their removal from the site, offering long term conservation. Constitutive apoptosis of circulating neutrophils is also paramount, serving a critical role for the balancing of neutrophil number and function.

The main characteristics of neutrophil apoptosis are externalisation of phosphatidylserine (PS), DNA fragmentation, a loss of mitochondrial membrane potential and membrane blebbing (Denecker *et al.*, 2000), the former being particularly key for efferocytosis to follow. Initiation of neutrophil apoptosis occurs in one of two ways, either via the intrinsic or extrinsic signalling pathways as outlined below and summarised in figure 3.

For the intrinsic pathway to be activated an imbalance between intracellular apoptotic factors must occur, specifically a shift from anti-apoptotic to pro-apoptotic. Indeed, whether the cell will survive or undergo apoptosis in response to internal stimuli, such as a lack of growth factors, DNA damage or biochemical stress, is a balance between groups of molecules within the Bcl-2 family. Bcl-2 family members reported to be present in human neutrophils include the apoptosis suppressing proteins Mcl-1 and A1/Bfl-1 as well as apoptosis potentiating proteins Bax, Bad and Bak (Moulding *et al.*, 2001). The function of these proteins is controlled by their localization, conformation, and phosphorylation state. The activation of Bax and Bak are critical in apoptosis regulation through the intrinsic mitochondrial pathway, however the presence of Mcl-1 and A1 respectively protects against Bax and Bak mediated cell death (McCracken and Allen, 2014). Loss of Mcl-1 or A1 allows Bax and Bak to oligomerize and form pores in the outer mitochondrial membrane, resulting in the release of cytochrome C from mitochondria into the cytosol, followed by the actions of caspase 9 and 3 (McCracken and Allen, 2014). For the extrinsic pathway, caspase 8 activates caspase 3 via receptors expressed on the surface of neutrophils. These ‘death’ receptors respond to soluble proteins, specifically those related or belonging to the tumour necrosis factor (TNF) family, such as Fas ligand (Fas-L), TNF-related apoptosis-

inducing ligand (TRAIL) and TNF-α itself (Kumar *et al.*, 2005). Regardless of the chosen pathway caspases (cysteine-aspartic proteases) play an essential role in all forms of cell death and inflammation, with caspase activation ensuring that cellular components are degraded in a controlled manner.

Despite being a tightly regulated process neutrophil apoptosis can be modulated by a number of factors resulting in its acceleration, deceleration or at times complete derailment. The inflammatory microenvironment can provide neutrophils with both survival and pro-apoptotic signals. For example neutrophil lifespan can be extended and/or apoptosis suppressed in response to granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin-8 (IL-8), bacterial components (e.g. LPS) and other pro-inflammatory mediators (Klein *et al.*, 2000, 2001; Andrew S Cowburn *et al.*, 2002; Glynn *et al.*, 2002; Martins *et al.*, 2010). In addition, *in vitro* studies have shown that treatment with glucocorticoids delays neutrophil apoptosis (Cox, 1995) as do certain environmental conditions, such as hypoxia (Hannah *et al.*, 1995; Murray *et al.*, 2003). Interestingly, in a severely hypoxic environment (~1% $O_2$) the ability of glucocorticoids to delay neutrophil apoptosis is lost, furthermore the treatment of neutrophils with glucocorticoids in severely hypoxic conditions was able to override the pro-survival effect of GM-CSF, resulting in increased apoptosis (Marwick *et al.*, 2013). Importantly, this suggests a potential therapeutic mechanism to augment the hypoxia-mediated delay in apoptosis at an inflamed site (Marwick *et al.*, 2013).

Accelerated activation of apoptosis may occur following neutrophil exposure to viral and bacterial pathogens, as well as in response to their phagocytosis. Indeed, both

fragment crystallisable region receptor (FcR) and complement receptor (CR) mediated phagocytosis has been demonstrated to promote programmed cell death, with apoptosis induced earlier following the engagement of both FcRs and CRs as opposed to either receptor alone (Kobayashi *et al.*, 2002). Neutrophil apoptosis has also been shown to be regulated via the β2 integrin Macrophage-1 antigen (Mac-1), in contrast to the suppression of apoptosis following engagement of ICAM-1, fibrinogen and myeloperoxidase, Mac-1 mediated bacterial phagocytosis increases neutrophil apoptosis (El Kebir and Filep, 2013). Additionally, and perhaps more obviously, reactive oxygen species, cellular survival/death signalling pathways and mitochondria have also all been implicated to have a role in influencing apoptosis.

As a consequence of neutrophil apoptosis changes in cell morphology arise, specifically the cell shrinks, chromatin compacts and the characteristic multilobular nucleus is lost. Additionally, functional properties are altered, whereby apoptosis of neutrophils disables their activation pathways and consequently results in the loss of their ability to operate as usual, particularly regarding chemotaxis, degranulation and respiratory burst. Furthermore, a decrease in the epitopes for immunoglobulins and surface receptor expression occurs and consequently competency in binding ligands is compromised (Dransfield *et al.*, 1994, 1995; Hart *et al.*, 2000).

During apoptosis, the typical symmetry of the plasma membrane phospholipids is lost, and PS exposure occurs as a result of its translocation from the inner to the outer membrane. Usually in a healthy cell, phospholipids are arranged asymmetrically in the plasma membrane. The negatively charged phospholipids, PS, along with phosphatidylethanolamine (PE), face the inner cytosolic side of the cell. These two

amine-containing phospholipids are positioned and maintained by ATP-dependent aminophospholipid translocases, called flippases, which move lipids from the exoplasmic to the cytosolic face. Whereas the outer exoplasmic membrane is rich in the positively charged phospholipids, phosphatidylcholine (PC) and sphingomyelin, which are moved in the reverse direction (cytosolic to exoplasmic) by floppases which are another type of ATP-dependent aminophospholipid translocase (Hankins *et al.*, 2015). In addition, ATP-independent enzymes, known as phospholipid scramblases, non-specifically and bidirectionally translocate phospholipids between the inner and outer membrane leaflets, randomising lipid distribution (Sahu *et al.*, 2007). When apoptosis is underway activated caspases cleave flippases and simultaneously activate scramblases. The capacity of scramblases to translocate PS rapidly and the absence of any counter movement by flippases results in efficient and expeditious PS exposure.

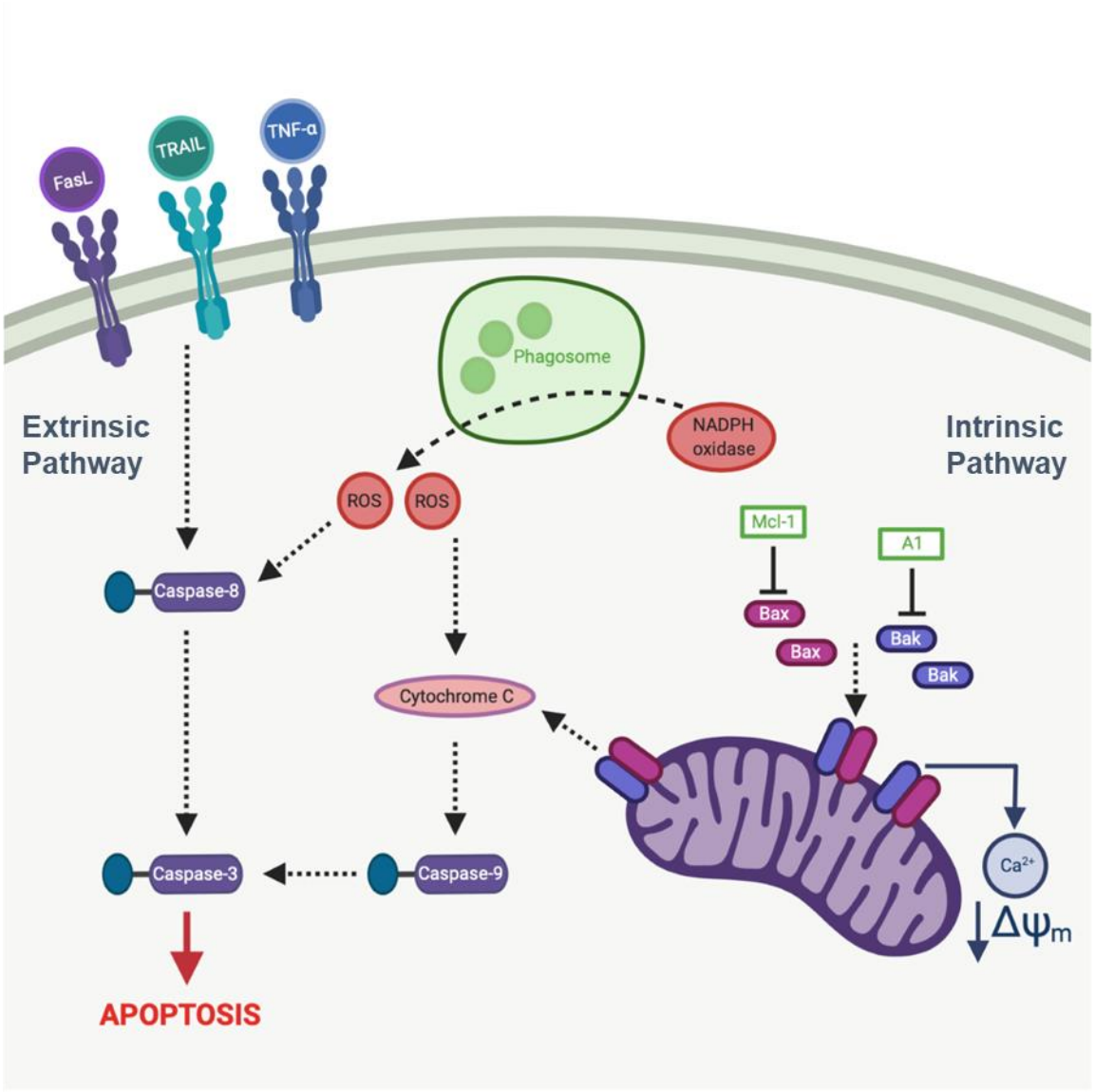



**Figure 3. Overview of neutrophil apoptosis pathways.**
Initiation of neutrophil apoptosis occurs either via the intrinsic or extrinsic signalling pathways. For the intrinsic pathway to be activated an imbalance between intracellular apoptotic factors must occur. The proapoptotic Bcl-2 family members Bax and Bak become more abundant than their anti-apoptotic counterparts Mcl-1 and A1, and their oligomerisation is no longer blocked. The joining of Bax and Bak at the mitochondrial membrane results in pore formation and a decrease in mitochondrial membrane potential (ΔΨm) allowing the release of cytochrome C and calcium ions into the cytosol. Cytochrome C activates caspase-9 which signals downstream to caspase-3. Following particle uptake, the formation of the phagosome results in NADPH oxidase derived ROS release, which acts on cytochrome C and initiates the intrinsic apoptosis pathway. The extrinsic pathway is initiated by 'death' receptors responding to soluble proteins (Fas-L, TRAIL and TNF-α). Ligand receptor binding signals activation of caspase-8, which signals to caspase-3. In all cases the activation of casapase-3 results in its cleavage and marks the final intracellular step of apoptosis. [Adapted from (McCracken and Allen, 2014), created using BioRender illustrating tool].

### *1.2.4. Neutrophil Clearance*

Both professional and non-professional phagocytes recognise PS on apoptotic cells as an ‘eat me’ signal. Furthermore, some professional phagocytes have the ability to bind PS either directly via a cell surface receptor or indirectly through a secreted protein able to bind PS as well as their own membrane protein (as discussed in (Segawa and Nagata, 2015)). These different cell surface and soluble ligands are discussed in more detail later, importantly for the successful resolution of inflammation these various recognition ligands, bridge molecules and receptors play a key role in determining inflammatory response and mediator production. In any case, the exposure of PS is key for the recognition of a dead cell by a phagocyte and serves as a fundamental ‘eat me’ signal. The engulfment of apoptotic cells follows, an action performed primarily by macrophages but also to a lesser extent by dendritic cells epithelial cells and mesenchymal cells. Of the aforementioned cells, macrophages, which are present throughout (almost all) animal tissues, are broadly regarded as the most competent phagocyte. Thus, the focus for the remaining text will be on macrophage phagocytosis of apoptotic neutrophils termed efferocytosis.

Macrophage ingestion of damaged host cells was first observed by Elie Metchnikoff in the late nineteenth century, however, this behaviour was for many years referred to simply as ‘physiologic inflammation’ and it wasn’t until the twenty-first century that the term ‘efferocytosis’, latin for ‘to carry to the grave’ was used for this action (deCathelineau and Henson, 2003).

Efferocytosis serves as the penultimate step in cell clearance (a process completed when the phagocyte transports the ingested cellular material away from the site).

Clearance of apoptotic cells in this manner is a necessity for the resolution of inflammation and is a behaviour that confers tissue protection by preventing the over activation of the innate immune response and consequent development of chronic inflammatory diseases, such as arthritis (as discussed in (Nagata *et al.*, 2010)). At the site of inflammation phagocytic removal of dead/dying neutrophils by macrophages prevents exposure to their toxic contents. However, when there is an overwhelming level of apoptosis greater than macrophage capacity or a direct impairment to the efferocytosis mechanism, macrophages may fail to intervene and clear the apoptotic cells (Silva *et al.*, 2008). In this way apoptotic neutrophils that fail to be cleared are suggested to undergo a process of autolytic disintegration, termed as secondary necrosis, involving activation of self-hydrolytic enzymes, cell swelling and irreversible damage to the cytoplasmic membrane (Silva *et al.*, 2008). During this process specific autoantigens undergo additional proteolytic degradation and the subsequent rupture of the neutrophil plasma membrane facilitates the release of these intracellular damage associated molecular patterns (DAMPs) (Wu *et al.*, 2001; Sachet *et al.*, 2017). These post apoptotic remnants and fragments may in turn stimulate autoantibody responses under proinflammatory conditions, thus perpetuating the inflammatory response (Mũnoz *et al.*, 2010) (and reviewed in (Rovere *et al.*, 2000)).

In many contexts the safe removal of apoptotic cells by macrophages is critical for the regulation of immune responses. In addition to the advantage of eliminating apoptotic cells from the local environment efferocytosis by macrophages modulates their mediator release and cell killing, restores tissue homeostasis and further co-ordinates adaptive immune responses. As such, inflammation is suppressed by increased release of anti-inflammatory mediators such as IL-10, TGF-β and $PGE_2$ and decreased

TNF-α. Tissue remodelling is controlled by phagocyte directed cell killing via modulated release of nitric oxide (NO) and death-inducing cytokine CD95 (Apo-1/Fas) ligand and regulated presentation of peptides by major histocompatibility complex class I (MHC-I) and class II (MHC-II) (as reviewed in (Savill and Fadok, 2000)).

Macrophages utilise cell surface receptors and/or secreted proteins to detect both the 'find me' signals that first evoke them as well as the 'eat me' signals displayed on apoptotic cells. PS is broadly considered the most prominent 'eat me' signal on apoptotic cells, however other less conspicuous signals exist (e.g. calreticulin). PS is recognised by many different cell surface receptors, direct receptors include phosphatidylserine receptor (PSR) (Li *et al.*, 2003), T cell immunoglobulin and mucin domain-containing molecule (Tim) -1, -3, -4 (Miyanishi *et al.*, 2007; Ichimura *et al.*, 2008; Nakayama *et al.*, 2009), CD300 (members a, b & f) (Choi *et al.*, 2011; Simhadri *et al.*, 2012; Murakami *et al.*, 2014), brain-specific angiogenesis inhibitor 1 (BAI1) (Park *et al.*, 2007), stabilin 1 and 2 (Park *et al.*, 2008, 2009), receptor for advanced glycosylation end products (RAGE) (Friggeri *et al.*, 2011) and lectin-like oxidized low-density lipoprotein receptor (LOX-1) (Murphy *et al.*, 2006). Whereas Tyro3, Axl, and MerTK (TAM) tyrosine kinase receptors (Seitz *et al.*, 2007), integrin-αvβ3 (Hanayama *et al.*, 2002), CD36 (Tait and Smith, 1999), calreticulin and LRP1 (CD91) (Gardai *et al.*, 2005) are supported by bridging molecules.

Bridging molecules are proteins secreted from macrophages to bind PS and aid in apoptotic cell engulfment, the use of these different sets of molecules is governed by tissue specific conditions (Okabe and Medzhitov, 2014). Macrophage secreted proteins include growth arrest-specific 6 (Gas6) and protein S (ProS1), both of which

form bridges via the TAM (Tyro3, Axl, and MerTK) tyrosine kinase receptors (Nakano et al., 1997; Rothlin et al., 2015), thrombospondin which acts as a bridging ligand for CD36 (Savill *et al.*, 1992), complement component 1q (C1q) and mannose binding lectin (MBL) which bridge to calreticulin and LRP1 (CD91) (Ogden *et al.*, 2001) and milk fat globule EGF factor 8 (MFG-E8) which binds integrin-αvβ3 on phagocytes (Hanayama et al., 2002). Indeed, in the absence of MFG-E8 thioglycolate-elicited peritoneal macrophages exhibited impaired uptake of apoptotic cells (Hanayama et al., 2004), therefore indicating that these bridging molecules are both context specific and essential for facilitating efferocytosis. Importantly, oxidation of the phospholipids exposed on the apoptotic cell, resulting in oxidised PS (oxPS), has been suggested to enhance the binding of some of these receptors (as reviewed by (Matsura, 2014)).

In addition to ligand receptor binding interactions efferocytosis is influenced by both the local microenvironment and macrophage programming. Regarding the former factors in the cellular milieu readily impact on the readiness of efferocytosis, for example the availability of the required appropriate bridging molecules is of significant consequence. Additionally a number of different lipid mediators and proteins (or their derived peptides) have been recognised for their ability to enhance efferocytosis including Annexin 1 (Scannell *et al.*, 2007), lipoxins, protectins, resolvins and maresins (Serhan *et al.*, 2011). In contrast there are also proteins, such as TNF-α, that when present within the inflammatory microenvironment can inhibit apoptosis and consequently clearance (Michlewska *et al.*, 2009), similarly presence of oxidants has the same outcome (McPhillips *et al.*, 2007).

Macrophages conditioned by alternative activation towards an anti-inflammatory (M2) phenotype are more adept at engulfing apoptotic cells in comparison to their classically activated (M1 phenotype) counterparts. In this case efferocytic receptors and bridge molecules are upregulated in response to the activation of nuclear receptors (peroxisome proliferator-activated receptors (PPARs) (Majai *et al.*, 2007; Mukundan *et al.*, 2009) and liver x receptors (LXRs) (A-Gonzalez *et al.*, 2009)) by macrophages. It has also been shown that PS on the surface of apoptotic neutrophils has the capacity to drive macrophage expression of these nuclear receptors (Fernandez-Boyanapalli *et al.*, 2009) thus further supporting their role in efferocytosis.

Usual practice following recognition of apoptotic cells is their removal, whereby neutrophils and their debris are digested within the macrophage phagolysosome. The apoptotic cell engulfment process is proposed to take place in two separate PS dependent steps (Hoffmann *et al.*, 2001). First the ‘tethering’ step, where PS binding its receptors on the surface of the macrophage results in the apoptotic cell becoming tethered to the macrophage surface. Followed by the tickling/uptake step, where soluble proteins from the macrophage bind PS on the apoptotic cell, thus ‘tickling’ and activating the receptors on the phagocyte, subsequent downstream signalling results in the polymerisation of actin (via signalling through the GTPase Rac1) and the uptake of the apoptotic cell (Nakaya *et al.*, 2008; Schlam *et al.*, 2015).

From what is known about the process of efferocytosis and the mechanisms that facilitate it, it is apparent that apoptotic cells and phagocytes can collaborate to carefully orchestrate the ingestion of the former by the latter. However, there are many aspects of the process that remain elusive and although many molecules have been

proposed to play a role, many of those that have been identified are yet to be comprehensively confirmed.

Macrophages change phenotype following efferocytosis. Whether apoptotic neutrophils act as an M2 differential signal and if the macrophage that comes in to contact with them adopts a ‘true’ M2 phenotype is yet to be ascertained. One proposed theory is that ‘M2-like’ macrophages undergo a sort of metamorphosis, altering their surface molecule expression to specifically become CD11b low upon interaction with apoptotic cells (Ariel and Serhan, 2012). The protein profile becoming increasingly pro-resolving in preparation for ingestion of apoptotic cells provides the rationale behind this modulation. Furthermore, macrophage treatment with resolvins and glucocorticoids was shown to reduce the apoptotic leukocyte ingestion required for CD11b low macrophage generation, indicating that macrophages may develop a ‘satiated’ phenotype (Schif-Zuck *et al.*, 2011).

Efferocytosis is generally considered to promote macrophage resolution programmes. One study that investigated directed migration in resolving macrophages showed that efferocytosis induces different chemokine receptors on macrophages (specifically upregulating CXCR4) (Angsana *et al.*, 2016) thus directly influencing cell trafficking. In any case efferocytosis has many immunomodulatory properties that further emphasize the significance of this process in inflammation related diseases. However, there is a need to better understand the pathways that regulate and enhance efferocytosis to return tissue to catabasis.

### *1.3. Rheumatoid Arthritis*

Inflammatory arthritis (IA) covers a range of severe pathological medical conditions, which primarily affect the body's immune system, included within these is Rheumatoid Arthritis (RA), ankylosing spondylitis and psoriatic arthritis. The inflammation of the joints (and other tissues) that is characteristic of these conditions results from the long-term persistence of an abnormal immune response and consequently classifies them as Chronic Inflammatory diseases (CID) (Dorland, 1980). The globally high prevalence of CIDs (Cicchitti *et al.*, 2015) and the debilitating nature of the conditions stresses the importance of elucidating the fine details of the immunological pathways that result in RA developing and its mechanisms of maintenance. Identifying such details is paramount to the discovery of how to manage RA and develop suitable therapies.

RA is the most common inflammatory joint disorder in the UK, with upwards of 400,000 sufferers within the UK population (Arthritis Research UK, 2018). Around three quarters of diagnoses are made in female patients and onset, regardless of gender, is typically between 40 and 50 years of age (Symmons *et al.*, 2002). However, RA affects all ages and overall, there is a growing number of patients with a condition that generally worsens symptomatically with duration.

#### *1.3.1. Manifestation*

The characteristic symptoms of RA are a result of an ongoing inflammatory response being led by abnormal feedback from the immune system. Principally this affects the joints yet the chronic and systemic nature of the disorder is reflected throughout the body and symptoms of the disease can occur in a number of tissues and organs (skin,

blood vessels, heart, lung and muscles) (Prete *et al.*, 2011). The severe effect on the joints is primarily the result of proliferative inflammation of the synovium commonly causing pain, swelling, oedema, erythema and stiffness. Long term affliction can result in a loss of bone density and architecture resulting in joint destruction (Nandakumar and Holmdahl, 2006). Although RA can cause problems in any joint anatomically, the small diarthrodial joints of the hands, feet, wrists and ankles are most affected, often in a symmetrical manner. As the disease becomes more active and severity increases additional intra-articular damage often manifests. Likewise, extra-articular problems with other tissues and organs within the body are more frequently observed (Cojocaru *et al.*, 2010), as well as fatigue, weight loss and formation of firm local tissue swellings, known as rheumatoid nodules, that occur close to the inflamed joint.

The most severe morphologic alterations that result from RA manifest in the joints. The primitive phase of this damage is characterised by oedema in the synovium, meaning an abnormal accumulation of tissue fluid occurs in the interstitial spaces of the connective tissue that lines the inner surface of the synovial joints. Consequently hyperplasia, enlargement caused by an increase in cell proliferation and thus amount of tissue, occurs and the synovium becomes thicker often losing its previously smooth contour (Rooney *et al.*, 1988). One of the key affirmations of RA is growth of an abnormal layer of fibrovascular/granulation tissue known as pannus, which forms over the surface of the joint. Pannus formation is the result of inflammation and exuberant proliferation of the synovium resulting in a proliferative mass of thickened synovial tissue, composed primarily of inflammatory cells and synovial fibroblasts. Specifically, pannus growth occurs over the articular cartilage and with progression will go on to invade and destroy cartilage and consequently erode bone (Yin *et al.*, 2002). As

disease progresses pannus formation may develop into ankylosis, initially of the fibrous form this can ossify and result in bony ankylosis.

Additionally, the inflammatory infiltrate invades the spaces surrounding the blood vessels within the synovial stroma, and increased vascularity results from the occurrence of angiogenesis (Elshabrawy *et al.*, 2015). Localised death occurs within regions of synovial tissue which for this reason is then shed into the joint to later be encased by fibronectin to form 'rice bodies' with a cartilage-like appearance (Jeevannavar and Baindoor, 2014). These free corpuscles float within the intra-articular space and may reach hundreds in number. In addition, neutrophils can accumulate in the synovium as well as within the synovial fluid (Mohr *et al.*, 1981).

Overall, this results in amplification of the disease process by gradually destroying collagen, decreasing joint space and causing damage to the bones of the joint. Bone erosion (via the actions of osteoclasts), thinning cartilage, stretched ligaments and tendons, and an inflamed synovium cumulate resulting in penetration of the synovium into the bone, decreased synovial fluid and swelling of the affected area. Furthermore, this deterioration of the joint structure is typically accompanied by inflammation, stiffness and pain (as discussed in (Guo *et al.*, 2018)).

A number of complications or comorbidities can occur as a result of having RA. Medical conditions regularly attributed with the illness include lung disease, cardiovascular (CV) disease and osteoporosis. Interstitial lung disease is reported in 10% of RA patients, (Bongartz *et al.*, 2010) and pulmonary problems result in 29% of early death in RA (Young *et al.*, 2006). However, the main cause of premature death

is CV disease which occurs in around 1 in 20 RA patients (Norton *et al.*, 2013; Dougados *et al.*, 2014). RA doubles the risk of heart attack (Solomon *et al.*, 2006) and increases the risk of stroke by 30% (Lindhardsen *et al.*, 2012) compared to the general population. Osteoporosis and therefore fragility fractures are also common amongst RA patients, with a reported 2-fold increase in the likelihood of the former (Michaud and Wolfe, 2007).

Both quality of life and working capacity are impacted for many, with 68% of individuals with RA being physically inactive (Platonova *et al.*, 1979). Frequently a vicious cycle commences whereby disease progression, increased pain and both physical and mental health influence one another. Increased levels of pain and functional disability in turn influences mentality in many cases, with major depression being reported to affect one in six (Matcham *et al.*, 2013). Despite the best research efforts being made to try to understand the disease the specific causes of RA currently remain unidentified. However, some factors including genetics, gender, age, weight and smoking have been acknowledged for influencing the risk of developing disease (Alamanos and Drosos, 2005).

The gene encoding the major MHC- II, DR beta 1 (HLA-DRB1) protein, which plays a central role in the immune system by presenting peptides derived from extracellular proteins, is the main genetic risk factor linked with RA (Kerlan-Candon *et al.*, 2001). It is presumed that the antigens capable of causing or accelerating joint disease (arthritogens) can bind to this shared epitope and initiate inflammatory synovitis. The gene encoding protein tyrosine phosphatase, non-receptor type 22 (PTPN22) protein, a lymphoid-specific intracellular phosphatase functioning in the T-cell receptor

signalling pathway, has also been proposed to be linked with RA. In this case specific polymorphisms of this gene are suggested to have a protective effect, reducing the risk of RA (Muñoz-Valle *et al.*, 2017). Opposingly, carriers of the major risk allele of PTPN22 have altered B cell receptor signalling resulting in increased self-reactive B cells causing breakdown of immune tolerance and autoimmune disease checkpoints (Menard *et al.*, 2011). Peptidyl arginine deiminase-4 (PAD-4), leading to increased citrullination of chemokines, as well as signal transducer and activator of transcription 4 (STAT4) and cytotoxic T-lymphocyte associated protein 4 (CTLA4), both involved in T cell activation, have also been proposed for genetic susceptibility to RA, however links are yet to be well defined (Verma and Sobha, 2015).

The process of disease initiation remains uncertain, however environmental factors have been implicated for their potential facilitation. The main environmental risk factor for anti-citrullinated protein antibody (ACPA)-positive RA patients is smoking (Perry *et al.*, 2014) due to increased citrullination in lung tissue, however being overweight also significantly increases the risk of disease. Risk increases by 15% from being overweight (body mass index (BMI) ≥ 25) and over 20% by being obese (BMI ≥ 30) (Yin *et al.*, 2002; Feng *et al.*, 2016). Gender and age also influence the likelihood of RA development, with the disease approximately three times more common in women than it is in men (Gabriel *et al.*, 1999; Riise *et al.*, 2000) and prevalence increases with age. Proposed microbial agents include Epstein-Barr virus (Trier *et al.*, 2018), Retrovirus (Tugnet *et al.*, 2013), mycobacteria (Winthrop and Iseman, 2013) and mycoplasma (Ataee *et al.*, 2015). These agents all share the ability to induce a loss of tolerance, however the modus operandi affiliating their induction of RA remains unexplained.

### *1.3.2. Pathogenesis*

RA is classified as an autoimmune disease, whereby aberrant behaviour of the body's own immune system results in healthy tissue being destroyed. The immune system normally maintains the tight regulation of the inflammatory response and a controlled defence system, however in the case of RA an erroneous response targets self-antigen instead of foreign. When this behaviour is sustained chronic inflammation ensues as an imbalance between pro-inflammatory and anti-inflammatory mediators manifests. In brief, a breakdown of immunological self-tolerance, induction of autoimmunity, chronic inflammation and consequently joint damage are favoured at the targeted sites (McInnes and Schett, 2007).

Numerous inflammatory cells and mediators are responsible for arthritis in the joint. It is a multistep, multicellular process that includes infiltration of lymphocytes and granulocytes into the articular cartilage as well as proliferation of synovial fibroblasts and macrophages resulting in an abundance of chemokines and proinflammatory cytokines (Mellado *et al.*, 2015). In addition to these cellular components (macrophages, dendritic cells, fibroblast-like synoviocytes, mast cells, eosinophils, neutrophils, T cells and B cells), cell surface molecules (adhesion molecules e.g. integrins), signalling components (ZAP70, PTPN22, JAK, mitogen activated protein kinase and Stat1) and humoral mediators (antibodies, cytokines, chemokines, metalloproteinases, serine proteases and aggrecanases) interact to drive the inflammatory response and therefore disease progression (Nandakumar and Holmdahl, 2006). Certainly, in RA the presence of autoantibodies such as rheumatoid factor (RF) and ACPA are highly associated with disease activity and progression (De

Rycke *et al.*, 2004). Likewise, it is the persistent participation of all these molecules, particularly increased expression of adhesion molecules and chemokines in the endothelium, which facilitates leukocyte infiltration to the site of inflammation. In the case of RA, continuous inflammation is targeted mainly to the synovium, providing the location where many cell types are activated and transformed (Harris, 1986).

Activated neutrophils are found in large numbers within both RA synovial fluid and pannus (as discussed in (Wright *et al.*, 2014)). Their release of cytotoxic products has long been thought of as their key contribution to disease, however it is now recognised that they have a more active role in regulating the functions of other immune cells (Mantovani *et al.*, 2011). The manner in which they do this has been categorised into four main ways: secretion of cytokines and chemokines, upregulation of plasma membrane receptors, direct cell-cell interaction and release of proteases (Wright *et al.*, 2014). Many differences in neutrophil phenotype can be detected in RA compared to healthy individuals, mainly that they are already primed for ROS production, have increased cytokine and chemokine secretion, and an extended lifespan (Raza *et al.*, 2006) courtesy of a hypoxic environment and presence of anti-apoptotic cytokines (GM-CSF and TNF-α).

A considerable contribution towards RA pathogenesis is also provided by an autoimmune reaction in which T cells play a pivotal role. High levels of activated CD4+ T cells will appear within affected joints very early in disease and are necessary for disease initiation (Wong *et al.*, 2006). The additional presence of Th17 cells, which induce the migration and activation of neutrophils and monocytes, and interferon-γ producing Th1 cells follows (Mellado *et al.*, 2015). Furthermore, the secretion of

cytokines by T cells is paramount for development of disease by stimulating synoviocytes and macrophages to produce additional pro-inflammatory mediators, which in turn drives the inflammatory response of other immune cells including their recruitment, survival and clearance. For example, endothelial cells in the synovium are activated resulting in enhanced leukocyte adhesion and transmigration.

Production of cytokines by fibroblast like synoviocytes (FLS) contributes to inflammation and cartilage destruction in RA, an imbalance between cell proliferation, survival, and death in the synovium results in increased proliferation of FLS with an aggressive phenotype encourages extracellular matrix invasion and increased deterioration of the joint (Bartok and Firestein, 2010). In addition, increased production of cartilage matrix metalloproteinases (MMPs) results in further destruction of articular cartilage.

Numerous pro-inflammatory mediators are found within synovial fluid that sustain the inflammatory response. The most prominent of the cocktail of inflammatory mediators produced are TNF-α, IL-1, and IL-6, which all have autocrine, paracrine and endocrine effects and account for many systemic manifestations, including upregulation and induction of additional cytokines. Interferon-γ, IL-17 (osteoclast activation), IL-23 (Th17 differentiation), IL-8 (cellular recruitment), IL-15 (T cell proliferation), $PGE_2$, GM-CSF and TGF-β are all known to be included within synovial fluid milieu (van Hamburg and Tas, 2018). Of these, the pro-inflammatory cytokine TNF-α has been shown to have a particularly crucial role (Tracey *et al.*, 1988), with levels in the synovium positively correlating to the degree of inflammation and bone erosion (Neidel *et al.*,

1995), consequently TNF-α blockade is currently a key therapeutic strategy used to treat RA (see section 1.3.4. and review by (Tracey *et al.*, 2008) for further detail).

### *1.3.3. Role of Neutrophils*

Neutrophils are recognised as having an active role in both the progression and persistence of inflammation. As a result of their persistent recruitment and prolonged survival, neutrophils are the dominant cell type found within the synovial joint, accounting for ~90% of the $2x10^9$ cells in a typical RA knee joint (Cross *et al.*, 2005), Compared to when circulating in the blood, neutrophils in the rheumatic joint display a more activated phenotype with increased cytokine release, NET formation and an enhanced capacity to produce ROS. Furthermore, and most relevant to this study, the presence of pro-survival factors in the joint affords the neutrophils a prolonged life span as a result of delayed apoptosis. Taken together, the on-going influx, increased release of cytotoxic and immunoregulatory molecules and the lack of apoptosis and clearance of neutrophils are potent drivers in both pathogenesis and perpetuation of RA.

In the RA joint the presence of inflammatory mediators, including soluble ICs (such as RF and ACPA) and cytokines, stimulate synovial fluid neutrophils. As a consequence of their activated or primed state these neutrophils release high levels of cytotoxic products (Robinson *et al.*, 1992). At the pannus, activated neutrophils respond to insoluble ICs, these are generally removed by phagocytosis however when engulfment fails to be successfully completed, frustrated phagocytosis results, intracellular ROS is generated and released along with hypochlorous acid (HOCl) (Fossati *et al.*, 2002; Cross *et al.*, 2005). Together, the enhanced secretion of oxidant,

ROS and granule enzymes, including cathepsin G, elastase and gelatinase, act to override protective agents within the joint and tissue damage ensues.

Large numbers of activated neutrophils are found in the pannus and synovial fluid within RA joints (Mohr, 1995; Cross *et al.*, 2005; Wittkowski *et al.*, 2007), consequently their release of inflammatory mediators provides an immense local effect. Beside their release of ROS and granule enzymes, which directly contributes to tissue damage, activated neutrophils also generate and release an array of additional cytokines and chemokines. Most notable of these include the cytokines IL-1β, IL-12, IL-18, IL-23, and TNF-α (plus TNF ligand superfamily member (RANKL) and TNFSF13B (BLys or BAFF)) and the chemokines CCL-2, CCL-4, CCL-5, and CXCL-8 (IL-8) (as reviewed in (Rosas *et al.*, 2017)). As well as IL-8, $LTB_4$ is also produced by activated neutrophils and the subsequent release of these chemoattractants into the joint directly recruits neutrophils. Recruitment of neutrophils to the inflammatory site is further boosted by the increased endothelial expression of ICAM-1 and IL-8 in response to neutrophil cytotoxic mediators and the enhanced adhesion of neutrophils to endothelial cells mediated by RF (Rollet-Labelle *et al.*, 2013). Indeed, the addition of neutrophil products to the existing inflammatory milieu is a key influence in the localised shift towards an aggravated and pro-inflammatory environment.

Activated neutrophils in the synovial joint also differ by their expression of cell surface receptors. It has been shown that neutrophils from the synovial fluid of RA patients synthesize and express large amounts of MHC-II molecules (Cross *et al.*, 2003). Interestingly, MHC-II was not detected on the surface of peripheral blood neutrophils from patients with RA nor were co-stimulatory molecules expressed by synovial fluid

neutrophils, suggesting that a novel interaction involving antigen presentation to T cells may occur within the RA joint (Cross *et al.*, 2003). Additionally, neutrophils from synovial fluid of RA patients express high affinity FcγR (CD64) on their cell surface, thus indicating that these neutrophils may have an altered ability to respond to immunoglobulin (IgG) containing ICs (Quayle *et al.*, 1997). Furthermore, blocking the FcγR has been shown to prevent development of disease in a neutrophil driven model of RA (Tsuboi *et al.*, 2011; Sadik *et al.*, 2012), therefore demonstrating the importance of this receptor on RA synovial fluid neutrophils, indicating that it serves a key role in disease progression.

Another augmented behaviour of neutrophils from patients with RA is their heightened propensity to release NETs (Khandpur *et al.*, 2013). NET formation requires the citrullination of histones, however in neutrophils from patients with RA this process occurs aberrantly resulting in the citrullination of additional proteins, such as vimentin (Khandpur *et al.*, 2013). Not only can this atypical citrullination result in the generation of neoantigens and thus new targets during epitope spreading (Kidd *et al.*, 2008), but the release of these citrullinated residues will stimulate ACPA production, sustaining the inflammatory response. Indeed, citrullinated vimentin has been found contained in NETs and in ICs from the synovial fluid of ACPA+ RA patients (Van Steendam *et al.*, 2010; Khandpur *et al.*, 2013), thus indicating NETosis as the release mechanism. Furthermore citrullinated vimentin is a prominent citrullinated antigen in ICs from synovial fluid of RA patients (Van Steendam *et al.*, 2010), therefore its release in NETs may be imperative for disease pathogenesis in ACPA+ RA patients. Certainly, a considerable increase in the titre of autoantibodies against citrullinated proteins is seen shortly before the onset of disease and continues to positively correlate with

progression (Lundberg *et al.*, 2005). In addition to citrullinated peptides NETs also contain chromatin (DNA and histones), cytoplasmic proteins and granule enzymes (myeloperoxidase, elastase, lactoferrin, MMP-9) (Urban *et al.*, 2009; Parker and Winterbourn, 2013). Upon NETosis these contents will be expelled with the latter having a particularly cytotoxic effect and further contributing to proliferation of disease and destruction of the collagen matrix within cartilage (Baici *et al.*, 1982; Van den Steen *et al.*, 2002). Furthermore, it has been demonstrated that cytokines contained within the inflammatory milieu, such as IL-17 and TNF-α, induce NETosis in RA neutrophils and that the sera from patients with ACPA+ RA stimulates healthy neutrophils to undergo NETosis (Khandpur *et al.*, 2013). In this manner, neutrophil release of NETs is a self-perpetuating action within the inflammatory environment of the RA joint.

The extended lifespan afforded to these neutrophils further evidences the derailment of a usually well-regulated innate immune response. For the resolution of inflammation, apoptosis of neutrophils must occur in an effective manner and their safe removal co-ordinated by subsequent mechanisms of efferocytosis. Impaired clearance in the inflammatory joint, means that when these neutrophils do eventually undergo spontaneous apoptosis, there is prolonged presence of apoptotic cells in the joint. Consequently, the autoantigens expressed on the surface of apoptotic neutrophils can lead to increased autoantibody production, thus further sustaining the inflammatory response.

Dampening down of over exuberant inflammatory actions of neutrophils may provide a promising target for therapeutic intervention for RA. Indeed, many current therapies

exert inhibitory effects on some neutrophil inflammatory functions including adherence, oxidant production, enzyme activity, degranulation and apoptosis (as discussed in (Wright *et al.*, 2010)). However, these therapies are not neutrophil specific nor are they purposed to have suppressive effects across multiple neutrophil inflammatory actions, suggesting that targeting neutrophils at the site of inflammation remains a relatively unexplored therapeutic approach. Certainly, evidence for the inflammatory role of neutrophils in RA is growing and it is becoming increasingly more apparent that their mechanisms of action in this inflammatory condition extend past their innate role in disease initiation and subsequent release of cytotoxic mediators. Thus, the direct targeting of neutrophils in the joint provides a novel therapeutic approach with the potential to influence their numerous downstream actions with roles in sustaining disease in RA.

### *1.3.4. Therapies*

For adults with newly diagnosed active RA the first line of treatment is usually monotherapy with a disease-modifying anti-rheumatic drug (DMARD), typically methotrexate (Kristensen *et al.*, 2006). Methotrexate is a conventional synthetic DMARD (csDMARD) and along with other members of the group is a slow-acting anti-rheumatic drug (SAARD). In contrast, biologic DMARDs (bDMARDs) which contain polypeptide structures (such as monoclonal antibodies) produce their effects more quickly. Targeted synthetic DMARDS (tsDMARDs) are the third major group of DMARDs and are a comparatively new group comprised of low molecular mass drugs which target specific processed, such as Janus kinase (JAK) and phosphodiesterase 4 (PDE4) inhibitors. As such DMARDs, either in the form of monoclonal antibodies against the cytokine (or its receptor) or soluble receptors serving as decoys, act by

targeting cytokines in the extracellular environment. In any case DMARDs function to decrease inflammation, slow down joint damage and ease the systemic effects of RA and other inflammatory diseases.

Therapy with a csDMARD, such as methotrexate, results in sustained disease-free remission in only 15% of RA patients (Van Der Woude *et al.*, 2009). When remission or low disease activity is not achieved from DMARD monotherapy or is not maintained the step-up strategy is combination treatment. Combination treatment may involve additional DMARDs, glucocorticoid treatment or anti-TNF agents. One example is that a csDMARD can be combined with a bDMARD, with a widely advocated combination being methotrexate and an anti-TNF agent (Emery *et al.*, 2008, 2010). There are three main TNF-blocking drugs which function as biologic agents to dampen down the inflammatory process: these include two monoclonal antibodies (adalimumab and infliximab) and a recombinant TNF receptor (etanercept) (Yamanaka, 2015). Despite this drug diversity therapeutic efficacy is not seen in all cases and around 30% of RA patients will be non-responders to TNF blockade (Hetland *et al.*, 2010).

Alternative therapies commonly used in the treatment of RA include tsDMARDs aimed at inhibiting the JAK pathway or bDMARDs targeting either specific cytokines or B cells, as well as a csDMARD directed at the proliferation of T helper cells.

JAK inhibition works by blocking the phosphorylation activity of one or more of the enzyme isoforms (JAK1, JAK2, JAK3 or Tyk2), consequently the related signal transducers and activators of transcription (STAT) proteins remain unphosphorylated, thus preventing their dimerization and translocation into the nucleus (Roskoski, 2016).

JAK enzymes bind to many different cytokine receptors and mediate pro-inflammatory cytokine, interferon, and hormone signalling, therefore by blocking phosphorylation of JAK a cluster of downstream inflammatory targets can be downregulated.

IL-6, which is released during acute inflammation by monocytes, macrophages and endothelial cells, is considered to be a key contributor to the shift from acute to chronic inflammation in RA and thus is a major mediator targeted by treatment strategies. IL-6 functions to stimulate hepatocyte production of CRP during the acute phase response. Furthermore, IL-6 interplay with B cells renders the cytokine important for many disease driving factors including B cell maturation, antibody production and Th17 differentiation. The IL-6 receptor is also expressed on neutrophils, with binding of the cytokine to its receptor resulting in the secretion of proteolytic enzymes and consequently cartilage degradation. Several studies have shown therapeutic efficacy of anti-IL-6 (Tocilizumab) treatment demonstrating significantly less worsening of joint damage and improved function (Maini *et al.*, 2006; Nishimoto *et al.*, 2007).

Inhibiting the cytokines IL-12 and IL-23 by use of a monocolonal antibody (Ustekinumab) against the p40 subunit common to both IL-12 and IL-23 blocks their interaction with the IL-12 Rβ1 receptor (to which they both bind). In this way the downstream actions of IL-12 and IL-23 on differentiation of naïve T cells to Th1 and Th17 cells respectively is inhibited and consequently so is inflammation. IL-12 and IL-23 blocking has been shown to be successful in psoriatic arthritis (Kavanaugh *et al.*, 2015), with studies in RA still lacking a definite conclusion of the efficacy on disease, nonetheless this treatment strategy appears to have an encouraging outlook for use in RA.

Inhibiting IL-17, an inflammatory cytokine produced by Th17 cells that plays a key role in bone destruction, is an additional promising approach to RA therapy. Indeed, RA patients show elevated levels of IL-17 in their serum and synovial fluid, thus decreasing the levels and/or functionality of this protein may be effective as lessening bone erosion and joint damage. Studies have so far been focused on psoriatic arthritis and psoriasis due to the significantly elevated levels of IL-17 in these diseases. Significant improvement has been seen using a monoclonal antibody against IL-17A (Secukinumab) in studies of psoriasis (Langley *et al.*, 2014) with a trend towards a positive response seen in RA (Kunwar *et al.*, 2016).

Another approach to RA therapy involves using anti-CD20 (Rituximab) to target B cells. B cells play a pivotal role in antigen presentation to T cells thus stimulating T cell activation and consequently accumulation in the synovium. However, by depleting CD20+ B cells autoantibody production, IC formation and B cell derived cytokines (such as IL-6 and TNFα) are reduced and thus synovitis is improved (Korhonen and Moilanen, 2010).

As well as B cell lymphocytes T cells may also be targeted. One mechanism of choice involves enzyme inhibition to limit proliferation of activated CD4+ cells. Specifically, inhibiting dihydroorotate dehydrogenase limits the rate of pyrimidine synthesis which is required for the accelerated proliferation of activated CD4+ cells commonly seen during progression of RA. Indeed, this is the primary mode of action for Teriflunomide (the active metabolite of the csDMARD Leflunomide) with secondary effects downregulating cytokine production, due to inhibition of NF-κB activation, and

decreasing production of MMPs, thus preventing joint destruction (Davis *et al.*, 1996; Kullich *et al.*, 2006).

Compared to other DMARDs, bDMARDs have a more rapid response and longer half-life. However interpatient variation, such as higher drug clearance or lower initial plasma concentrations of the target cytokine, means that some patients may respond to particular bDMARDs but not others, hence the requirement for a repertoire of target specific drugs. When successful these treatments, whether administered individually or in combination, are anti-rheumatic and thus improve quality of life, nonetheless there are still key issues surrounding their use. These include risk of infection, loss of efficacy, and primary non-response but above all limited long-term improvement (Emery, 2012) and the requirement for continual treatment for sustained control (Firestein, 2003).

Although current treatment strategies aim to intervene with the course of disease as early as possible the therapeutic response and resistance is significantly influenced by the clinical evolution of disease. Furthermore, inflammation of the synovium exhibits great heterogeneity with different disease pathotypes categorised by their cellular and molecular signatures. Specifically transcriptomic clustering has observed lymphoid-(L), Myeloid-(M) and two Fibroblast-(F,F/A) prevalent patterns when RA synovitis samples were assessed (Pitzalis *et al.*, 2013), findings which may explain the inconsistency in response to current therapeutics.

### *1.3.5. Animal Models*

Animal models of RA have made significant contributions to the current understanding of the disease. Not only have they advanced what is known about RA as a disease but many of the discoveries are translatable to other inflammatory diseases. Overall, these models have broadened our knowledge on the immune response and importantly on the interplay between the mediators and mechanisms driving inflammation and resolution. As previously discussed, RA is a complex disease involving inflammation, tolerance and autoimmunity as such the use of animal models can provide the advantage of a more simplified system (Choudhary *et al.*, 2018). Indeed, there are several models of RA that either develop spontaneously or can be induced and in either case the mechanisms driving disease and comparability to features of human RA is unique to each model. In this manner it is particularly important to carefully evaluate the choice of models when deciding which is most appropriate for investigating a specific research question.

Spontaneous development of arthritis occurs in only a few murine strains, namely Murphy Roths Large (MRL)/1 mice (Hang *et al.*, 1982), New Zealand Black mice (Rordorf *et al.*, 1982), tiptoe walking-Yoshimura (twy) mice (Hosoda *et al.*, 1981) and Sakaguchi (SKG) mice (Sakaguchi *et al.*, 2003). Models of 'spontaneous' RA are often the result of gene manipulation, common models of this type include TNF-α over-expressing transgenic mice that develop an erosive polyarthritis with a similar phenotype to human RA (Keffer *et al.*, 1991) and IL-1 receptor antagonist (IL-1Ra)-deficient mice, which along with arthritis develop additional auto-immune conditions, demonstrating that excess IL-1 signalling causes autoimmunity (Horai *et al.*, 2000). Importantly, TNF-α transgenic mice have helped to validate TNF-α as a dominant

cytokine in RA and have facilitated development of anti-TNF-α therapies (Li and Schwarz, 2003), as well as determining roles for IL-1 activation and non-specific signalling effects on T cells (Iwakura, 2002). Additional models include Human T Lymphotropic Virus Type-1 (HTLV-1) transgenic mice (Iwakura *et al.*, 1991), IL-1α transgenic mice (Niki *et al.*, 2001), TNF AU-rich element (ARE) deficient mice (Kontoyiannis *et al.*, 1999) and K/BxN mice (Kouskoff *et al.*, 1996), with the latter being discussed in more detail later.

Induced arthritis models that show similar pathology to human RA include serum transfer induced arthritis (STIA) (Wipke and Allen, 2001), adjuvant induced arthritis (AIA) (Pearson, 1956), streptococcal cell wall-induced arthritis (DeJoy *et al.*, 1989), pristane-induced arthritis (Potter and Wax, 1981), collagen antibody induced arthritis (CAIA) (Khachigian, 2006) and most notably the collagen-induced arthritis (CIA) model (Brand *et al.*, 2004). CIA is the most widely used animal model for RA and is largely considered the 'gold standard' in mouse models of the disease. Similar to human RA, CIA is characterized by synovial infiltration and swelling as well as cartilage and bone erosion (Asquith *et al.*, 2009). Induction of arthritis in this model involves immunization with collagen type-II (CII) in incomplete Freund's adjuvant (IFA) with the subsequent development of arthritis appearing 3–5 weeks later as a result of T cell recognition of CII and B cell production of CII specific autoantibodies (Rosloniec *et al.*, 1988). Thus, this model is dependent on T and B cells and the adaptive immune response to an active immunization, as such this model incorporates both the priming and effector phases of arthritis and is therefore the most similar model to human RA (Korganow *et al.*, 1999). However, this does not render alternative models redundant.

The K/BxN STIA model offers advantages over the CIA model; it can be used to solely study the mechanisms of the effector phase, it develops over days rather than weeks and it can be induced in multiple strains of mice (as reviewed in (Christensen *et al.*, 2016)). Disease induction by the passive transfer of autoantibodies in the K/BxN STIA model means that both T cells and B cells are dispensable. In this manner, the K/BxN STIA model is ideal for investigating the mechanisms of interaction between autoantibodies and components of the innate immune system in driving the progression of arthritis. Specifically, neutrophils have been shown to be an essential component in the inflammatory response in this model and their deficiency confers disease resistance to K/BxN STIA (Wipke and Allen, 2001). Importantly, the inflammatory response in this model is completely dependent on autoantibodies whereas the inclusion of CFA in the CIA model induces an additional innate immune stimulation (as discussed in (Christensen *et al.*, 2016)). Another significant advantage of this model over CIA is its broad application, as mentioned a wide range of strain backgrounds are susceptible to K/BxN STIA (Ji *et al.*, 2001), whereas CIA is limited to DBA/1, B10.Q, and B.10RIII, with C57/Bl6 mice developing only a mild arthritis (Rosloniec *et al.*, 1988). Taken together, the features described above make the K/BxN STIA the most applicable model for this study.

The K/BxN mouse model of arthritis (first reported in 1996 by Mathis and Benoist) involves generating K/BxN mice by crossing KRN mice (transgenic for the T-cell receptor (TCR) responsible for recognising ribonuclease peptide (RNase) 43-56) on a C57BL/6 background with non-obese diabetic (NOD) mice. The NOD mice are a strain genetically prone to autoimmune response due to homozygous expression of the MHC

class II allele A(g7), this genetic background allows them to act as the secondary component for a successful K/BxN crossbreed (Monach *et al.*, 2008).

The offspring that result (K/BxN mice) spontaneously develop the symptoms of severe arthritis post-weaning at 4-5 weeks of age (Kouskoff *et al.*, 1996). This manifestation has been shown to be the result of the KRN TCR recognising glucose-6-phosphate isomerase (G6PI), a peptide on a ubiquitously expressed self-antigen bound to NOD antigen-presenting cells (APCs). As a result of this recognition T cells become activated and interact with B cells promoting their activation as well as production of disease inducing IgGs (Christensen *et al.*, 2016). Presence of the antibody brings about IC formation and consequently activation of numerous innate immune cells. Anti-G6PI autoantibodies induce joint-specific inflammation from an autoimmune response that results in an inflammatory arthritis. The anti-GPI antibodies are detectable in the serum of K/BxN mice from the onset of disease and although initially low, the titre level rises until around 60 days old (Maccioni *et al.*, 2002). The transfer of K/BxN sera from these mice induces a robust arthritis in recipient mice (Korganow *et al.*, 1999; Ji *et al.*, 2001). Specifically the distal joints of the limbs, including the knee, ankle, tarsal and metatarsal regions of the hind limb along with the corresponding forelimb locations are the sites where inflammation will commonly manifest, with some regions of the spine also being mildly susceptible (Kouskoff *et al.*, 1996). A rapid response and highly robust disease incidence ensues, which is reproducible in a considerable number of mice strains, furthermore by modifying the serum dosing strategy the onset and manifestation of systemic disease can vary, consequently shaping the overall disease profile to fit specific experimental purposes. As such, K/BxN serum transfer is a well-utilised model of murine arthritis for many preclinical

studies with the model baring particular benefit for investigating the interaction of innate immune components involved in the progression of RA.

Transfer of K/BxN serum into recipient mice elicits a rapid immune response (Wipke *et al.*, 2002). The first step following intra-peritoneal (i.p.) administration of K/BxN serum involves autoantibodies entering the circulation, as such the early stage of disease initiation occurs in the blood where administered anti-G6PI autoantibodies bind to G6PI resulting in the formation of soluble ICs. Next, these newly formed complexes interact with neutrophils, whereby they bind to Fcγ-receptors (FcγR) on the cell surface, signaling the release of vasoactive mediators. In turn, vascular permeability is increased thus allowing for the diffusion of ICs and anti-G6PI antibodies out of the blood and into the perivascular tissue. Mast cell degranulation results in the release of additional vasoactive mediators further increasing vascular permeability. Consequently, the synovial membrane is easily penetrable allowing ICs and anti-G6PI antibodies (as well as additional non-specific antibodies and serum proteins) to enter the joint cavity. Once present in the joint the anti-G6PI antibodies bind G6PI antigens expressed on the articular surface, expression of which is significantly amplified on arthritic cartilage (Christensen *et al.*, 2016). In the K/BxN model this process occurs almost exclusively in the distal joints however it is yet to be determined why increased vascular leakage in response to circulating ICs is localised to the extremities of the limbs. Additionally it is worth noting that the interaction with FcγRs is essential during this initiation phase, evidenced in FcγR KO mice which were shown to be protected from developing arthritis in response to administration of K/BxN serum (Corr and Crain, 2002; Hampel *et al.*, 2013).

After the initiation phase is the effector phase. Here the progression of joint localised arthritis has a strong dependence on the continued interaction of anti-G6PI antibodies and ICs with innate immune cells. Within the joint cavity ICs activate the alternative complement pathway, here the hydrolysis of C3 (into C3a and C3b fragments) leads to the cleavage of C5 (by C3b, into C5a and C5b), a product of which (C5a) is able to bind neutrophils resulting in their activation. Complement activated neutrophils release leukotriene B4 ($LTB_4$) which upon interaction with neutrophils stimulates their release of interleukin-1β (IL-1β). IL-1β induces tissue cells to release chemokine C-X-C motif ligands 1 and 5 (CXCL1, CXCL5) and chemokine C-C motif ligand 9 (CCL9), as well as neutrophil release of CXCL2 and CCL3, thus stimulating increased neutrophil recruitment (Chou *et al.*, 2010). Additionally, presence of IL-1β mediates release of granulocyte colony stimulating factor (G-CSF) that acts to further mobilise neutrophils. These conditions and the activated endothelium trigger recruitment of leukocytes to the site, with the infiltrating cell population composed predominantly of neutrophils and a small contribution of monocytes ($Ly6C^-$) (Misharin *et al.*, 2014).

In addition to neutrophil infiltration additional immune cells present at the site of inflammation also have essential roles during this effector phase. Macrophages in the joint release macrophage inhibitory factor (MIF) which in combination with neutrophil released IL-1β mediates cartilage degradation (Jacques *et al.*, 2006). Release of MIF also contributes to receptor activator of nuclear factor κ B (RANK) interaction with its ligands on osteoclasts, resulting in their activation and therefore the breakdown and erosion of bone (Christensen *et al.*, 2016). Tumour necrosis factor (TNF) and $PGE_2$ are both produced in the joint and are considered to be responsible for conveying the

pain associated with inflammation (Christensen *et al.*, 2016), however whether this is a direct or indirect contribution is currently not well understood.

In any case, common sites of affliction in the model closely resemble RA in many aspects. The release of pro-inflammatory cytokines TNF-α, lL-1 and IL-6 in inflamed joints is seen in both RA and the K/BxN model. Furthermore IL-1 and TNF-α are particularly important for the development of arthritis (Lubberts and Berg, 2013). Mouse strains deficient in TNF-α have been demonstrated to have decreased incidence of disease whereas an absence of IL-1 completely protected mice from developing arthritis in response to serum (Ji *et al.*, 2002). IL-17, which is implicated in the pathogenesis of RA has also been proposed to have a role in the K/BxN model, however current findings for its role in the model are discrepant (Jacobs *et al.*, 2009; Katayama *et al.*, 2013). Increased levels of prostaglandins are also found at sites of inflammation in RA patients and have been shown to be similarly elevated in the K/BxN arthritic joint. Specifically, high levels of $PGE_2$ and 6-keto prostaglandin F1α (*6*-k-*PGF1α,* a stable hydrolyzed product of prostacyclin (PGI2)) are detected in the inflamed joint, however only the latter is essential for development of disease (Chen *et al.*, 2008). Integrins and their ligands are indispensable to the development of inflammatory arthritis in both human and mouse models. The $\beta_2$ integrins are particularly essential, with the CD11a/CD18 complex, also known as lymphocyte function-associated antigen 1 (LFA-1) and the CD11b/CD18 complex, referred to as both Macrophage-1 antigen (MAC-1) and complement receptor 3 (CR3) shown to be critical (Watts *et al.*, 2005). Recruitment of leukocytes, particularly neutrophils, into the joint has been shown to be dependent on expression of these integrins, with no

response to K/BxN serum in mice deficient for the $\beta_2$ integrins or following integrin blockade (Watts *et al.*, 2005).

The K/BxN serum transfer model of arthritis, in the absence of continual serum dosing, is a self-resolving disease, thus allowing the resolution phase to also be studied. As autoantibody levels drop the disease begins to abate, immune cell infiltration ceases and inflammation ameliorates. As testimony to disease presence the architecture of the afflicted joint remains altered for much of the initial resolution process, however as inflammation continues to resolve and mice return to baseline the remaining bone and cartilage that once appeared eroded and irregular has been demonstrated to repair (Matzelle *et al.*, 2012). A finding that provides significant benefit for research regarding reversal of inflammatory damage and the process of resolution.

In summary, the response generated mimics the immunological mechanisms occurring in the effector phase of RA, similarly characterised by the activation of innate immune cells (largely neutrophils and macrophages) as well as cytokines, chemokines, complement factors, Toll-like receptors, Fc receptors and integrins (Christensen et al., 2016). The presence of the G6PI self-antigen in both the disease model and RA is of prevalent importance. Responsible for sustaining disease in the K/BxN model the antigen is also present on the synovial lining and arteriole endothelial cell surface of RA patients alongside an elevated concentration of the anti-GPI immunoglobulin G (IgG) detectable in the serum and synovial fluid (Schaller *et al.*, 2001). Despite the mass similarities the autoantibody profile also exhibits variation between the model and the human disease, specifically Rheumatoid Factor (RF) has elevated presence in upwards of 70% of RA patients, yet is undetected in the mouse

model, whereas Immunoglobulin G (IgG) is raised in the mouse model and binds to organs, a behaviour not evidenced in RA. Even though these differences exist the K/BxN model is broadly considered to replicate human RA whereby swelling and joint destruction occurs as the result from the infiltration of immune cells, increased production of pro-inflammatory mediators and erosion of bone.

### *1.4. Galectins*

It is becoming increasingly apparent that many potential immunomodulators exist within the superfamily of lectins. Found within a diversity of organisms (Lam and Ng, 2011), lectins are carbohydrate binding proteins which branch into four distinct subgroups: C-type lectins (including the selectins); P-type lectins; pentraxins; and galectins (originally S-type lectins) (Barondes, Castronovo, *et al.*, 1994) with members of each group being determined by their binding specificity for β-galactoside sugars (Nishi *et al.*, 2005).

Encoded by the LGALS gene and sharing a characteristic amino acid sequence (Barondes, Cooper, *et al.*, 1994), there are currently 15 mammalian galectins that have been identified and named with consecutive numbers. Not all galectins have been identified in humans, of Gal-1 through to Gal-15, Gal-5 and Gal-6 are rodent exclusive, and Gal-11, Gal-14 and Gal-15 are found only in sheep and goat (Leffler *et al.*, 2002). Nonetheless galectin ancestry in primitive organs and their current presence across vertebrates, invertebrates and protists suggests integral behaviours for the family. However, this does not confer uniformed expression, indeed galectin expression (both intracellularly and extracellularly) is highly variable being either cell type specific or showing a more varied distribution, as detailed in table 1.

**Table 1. Galectin species and tissue distribution.**

| Galectin | Species | Tissue Distribution | References |
|---|---|---|---|
| Gal-1 | Vertebrates | Diverse | (Cooper, 2002) |
| Gal-2 | Birds, mammals, frogs, lizards | Gastrointestinal tract, placenta | (Hutter *et al.*, 2015)<br>(Oka *et al.*, 1999) |
| Gal-3 | Vertebrates | Diverse | (Cooper, 2002) |
| Gal-4 | Vertebrates (not birds) | Intestine and stomach, uterine epithelial cells, blood vessel walls, hippocampal and cortical neurons | (Froehlich *et al.*, 2012)<br>(Huflejt and Leffler, 2004)<br>(Velasco *et al.*, 2013) |
| Gal-5 | Rat | Lung, kidney, reticulocytes, bone marrow, erythrocytes | (Cerra *et al.*, 1985)<br>(Gitt *et al.*, 1998)<br>(Lensch *et al.*, 2006) |
| Gal-6 | Mice | Gastrointestinal tract, liver, kidney, spleen, heart | (Gitt *et al.*, 1998)<br>(Houzelstein *et al.*, 2013)<br>(Houzelstein *et al.*, 2008) |
| Gal-7 | Mammals | Gastrointestinal tract, stratified epithelia, skin, foetal heart | (Magnaldo *et al.*, 1998)<br>(Sato *et al.*, 2002)<br>(Timmons *et al.*, 1999) |
| Gal-8 | Vertebrates | Liver, kidney, cardiac muscle, lung and brain | (Hadari *et al.*, 1995) |
| Gal-9 | Vertebrates | T-lymphocytes, small intestine, liver, uterine epithelial cells, skin epidermis and oesophageal epithelium | (Froehlich *et al.*, 2012)<br>(Lensch *et al.*, 2006)<br>(Matsumoto *et al.*, 1998)<br>(Wada *et al.*, 1997) |
| Gal-10 | Primates and bats | Eosinophils | (Abedin *et al.*, 2003) |
| Gal-11 | Primates/ruminants | Placenta/abomasal mucosa and bile duct epithelial cells | (Dunphy *et al.*, 2000)<br>(Young *et al.*, 2012)<br>(Than *et al.*, 2009) |
| Gal-12 | Vertebrates | Adipose Tissue | (Hotta *et al.*, 2001)<br>(Yang *et al.*, 2004) |
| Gal-13 | Primates | Placenta | (Than *et al.*, 2004) |
| Gal-14 | Ruminants | Eosinophils | (Young *et al.*, 2009) |
| Gal-15 | Ruminants | Uterus | (Gray *et al.*, 2004) |

(Table adapted from (Johannes *et al.*, 2018))

Despite all galectins containing a conserved carbohydrate-recognition domain (CRD) of ~130 amino acids, the position of this domain within the overall structure of the protein varies, as does the inclusion of additional domain regions. On the basis of their structural differences the galectin family is subdivided into three groups, as shown in table 2, where each member is classified accordingly as either prototype, chimera-type or tandem-repeat-type (Yang *et al.*, 2008). Gal-1, Gal-2, Gal-5, Gal-7, Gal-10,

Gal-11, Gal-13 and Gal-14 belong to the prototype subgroup, possessing a single CRD and exist as either monomers (Gal-5, Gal-10 and Gal-14) or non-covalent dimers (Gal-1, Gal-2, Gal-7 and Gal-11) with the exception of Gal-13 which is unique in being stabilised by two disulphide bonds (Rapoport *et al.*, 2008). Chimera-type galectins consist of one CRD with the addition of an extended non-lectin N-terminal region, this structural organisation is unique to galectin-3, the only galectin classified as chimera-type. The third subgroup are the tandem-repeat-type galectins, which include Gal-4, Gal-6, Gal-8, Gal-9 and Gal-12. These five galectins are characterised by having two CRDs connected by a short linker peptide, with different sugar binding specificities pertaining to each domain. The two domains provide these galectins with the capability to form cross links with glycoconjugates in a more variable manner, resulting in more galectin-glycoconjugate combinations than the prototype or chimera-type galectins (Nishi *et al.*, 2005; Rapoport *et al.*, 2008).

**Table 2. Galectin family subgroups and structure.**

| Prototype | Chimera | Tandem-repeat |
|---|---|---|
|  |  |  |
| Single CRD<br>Monomer/Homodimer | Single CRD<br>Extended N-terminus | Two CRDs<br>Linker peptide |
| Gal-1, Gal-2, Gal-5, Gal-7, Gal-10, Gal-11, Gal-13 and Gal-14 | Gal-3 | Gal-4, Gal-6, Gal-8, Gal-9 and Gal-12 |

Across the galectin family the degree of similarity between the nucleotide sequences of the CRDs varies considerably. As such galectins show individual carbohydrate binding preferences for the small sets of oligosaccharides that build the larger glycoproteins, with further diversity occurring in valence number and from the ability of some galectins to bind multiple antigens/receptors. Regardless of these differences,

members of the galectin family discerningly form networks with multivalent glycoconjugates in a carbohydrate dependent manner. In addition to the ability to exert extracellular actions galectins also perform roles intracellularly. In such circumstances it is thought their behaviours are independent of carbohydrate binding instead interacting with intracellular ligands and facilitating signalling pathways accordingly.

Galectins do not contain a classical signal sequence, thus are not destined towards the secretory pathway. In spite of this lack of signal sequence for transport into the endoplasmic reticulum, members of the galectin family, localised mainly to the cytoplasm (and less frequently within the nucleus), are still able to exit cells via a non-classical pathway. The exact mechanisms remain poorly understood, however the secretion of galectins has been demonstrated to require their galactoside-binding activity (Seelenmeyer *et al.*, 2005).

Many members of the galectin family demonstrate low level endogenous expression across numerous different tissues and organs, with the immune system seen to exhibit the most abundance (Li *et al.*, 2013). Regarding their role in inflammation some immune cells, as well as vascular endothelial cells, have been shown to endogenously express several members of the galectin family. Although expression is relatively low under basal conditions levels are generally upregulated in response to cell activation. Recent studies have identified that galectins present at inflammatory sites have potent immuno-regulatory properties through both their extracellular and intracellular actions. Through binding to the surface of leukocytes, galectins mediate cell-cell and cell-matrix interactions consequently modulating recruitment and trafficking of leukocytes (Cooper *et al.*, 2008; Norling *et al.*, 2009). Another more extensively studied function

of galectins is their regulation of apoptosis, where findings have shown that particular cell types (including fibroblasts and T cells) are susceptible to galectin-induced apoptosis (Seki *et al.*, 2007; van Kooyk and Rabinovich, 2008). Taken together these findings provide evidence for a galectin-leukocyte interplay that may play a role in mediating various outcomes.

Four of the fifteen members of the galectin family (specifically galectin-1, -3, -8 and -9) demonstrate influential roles in inflammation. Gal-1 has been shown to inhibit leukocyte recruitment (La *et al.*, 2003; Cooper *et al.*, 2008; Norling *et al.*, 2008) with an anti-inflammatory effect involving the inhibition of both soluble and cellular mediators of the inflammatory response (Rabinovich *et al.*, 2000). Gal-3, Gal-8 and Gal-9 generally promote leukocyte recruitment (Norling *et al.*, 2009) with Gal-3 and Gal-8 evidencing pro-inflammatory effects whilst the principal behaviour of Gal-9 is still debatable. To perform their roles influencing leukocyte trafficking galectins must be present at the site of inflammation. However, in certain immune diseases circulating galectins are also detectable in the blood serum. Specifically, Gal-1 and Gal-3 are detectable in both the sera and synovial fluid of patients with RA. Expression of Gal-1 has been shown to be down regulated in the synovial fluid from RA patients (Xibillé-Friedmann *et al.*, 2013), yet markedly increased in the sera (Mendez-Huergo *et al.*, 2019). Levels of Gal-3 have also been reported to be increased in both the sera and synovial fluid from patients with RA (Ohshima et al., 2003). However, in an alternative study the opposite was shown, where in RA sera samples Gal-3 was down regulated (Mendez-Huergo *et al.*, 2019), thus further investigation is necessary.

### *1.4.1. Gal-1*

Gal-1 was first identified in the mid-1970s when it was found in various organs of three different species around the same time. Specifically it was detected in electric eel's electric organ tissue, in calf heart and lung tissue and in chick embryos (Teichberg *et al.*, 1975; de Waard *et al.*, 1976; Kobiler and Barondes, 1977; Nowak *et al.*, 1977), where in all cases the protein was noticed for its β-galactoside binding activity and its hemagglutinating effects on red blood cells. Highly conserved across species Gal-1 is encoded by the LGALS1 gene located on the 22q13.1 band of chromosome 22, the gene contains four exons and is 135 amino acids in length. Purification and crystallisation of the protein has revealed that the monomer structure with its single carbohydrate binding site, consists of two β-sheets, one of five strands and one of six strands, and that a 22-strand dimer is formed from an anti-parallel β-sandwich arrangement that results in the two binding sites being located on the same side of the molecule (Liao *et al.*, 1994). Upon dimerization a hydrophobic core is formed between the monomers, an interaction that is essential for maintenance of the structure.

### *1.4.2. Gal-1 in Inflammation and Resolution*

Galectins were first confirmed to act in inflammation following several studies in the early 1990's that revealed members of the protein family having a role in cell adhesion and migration (Cooper *et al.*, 1991; Colin Hughes, 1992). These findings followed a couple of much earlier studies describing a 14kDa β-galactoside binding protein (now known as Gal-1) with immunosuppressive effects in models of experimental autoimmune myasthenia gravis (EAMG) (Levi *et al.*, 1983) and experimental autoimmune encephalomyelitis (EAE) (Offner *et al.*, 1990). However, at this time

experimenting with the family of proteins was still particularly novel and so these early studies required further investigation and corroboration. Consequently, these effects seen in T cell mediated diseases led to increased attention being focused on the interactions of Gal-1 with T cells. Initial work revealed that thymic epithelial cells express Gal-1 which directly influences the adhesion of immature T cells (Baum, Pang, *et al.*, 1995). Furthermore, unlike mature and activated cells, immature T cells were not able to facilitate Gal-1 binding to the cell surface. As part of these initial Gal-1 studies the peripheral lymph nodes were also checked, here the same group showed that the protein is present in activated, but not resting, lymphoid tissue and that this expression was localised specifically to the high endothelial venules (Baum, Seilhamer, *et al.*, 1995). En masse these studies were strongly indicative of an immunomodulatory role for Gal-1.

Much of the focus of galectin research has continued to be directed by its interactions with T cells. More recent work has uncovered the ability of Gal-1 to target specific subsets of T cells (Cedeno-Laurent and Dimitroff, 2012) often inducing their apoptosis, a behaviour that is likely to lead to the protein's suppressive effects in several models of autoimmune disease (Rabinovich, Daly, *et al.*, 1999). Furthermore, Gal-1 has also been shown to inhibit trafficking of T cells through endothelial monolayers (Norling *et al.*, 2008) and have an effect on tumour modulation (Astorgues-Xerri *et al.*, 2014).

Compared with studies on T-cells, the functions of Gal-1 on cells of the innate immune system such as macrophages and neutrophils are comparatively fewer. Exogenous Gal-1 induces 12/15-lipoxygenase expression and promotes macrophage conversion to a pro-resolving phenotype that are quicker to reach efferocytic satiation (Rostoker

*et al.*, 2013). In general Gal-1 treated macrophages favour the alternative activation pathway (Correa *et al.*, 2003), resulting in a more M2 like phenotype (Abebayehu *et al.*, 2017), with reduced MHC-II expression (Barrionuevo *et al.*, 2007) and decreased release of pro-inflammatory mediators (Rabinovich *et al.*, 2000; Kogawa *et al.*, 2011). Correspondingly, endogenous Gal-1 is associated with mature ($CD11b^{high}$) macrophages but not pro-resolving ($CD11b^{low}$) macrophages (Rostoker *et al.*, 2013).

The majority of studies investigating the role of Gal-1 on neutrophils indicate that this protein is protective, including the ability to induce surface phosphatidylserine (PS) exposure, indicating a potential role in neutrophil clearance (Dias-Baruffi *et al.*, 2003; Stowell *et al.*, 2007, 2009), as well as a role in neutrophil extravasation (Cooper *et al.*, 2008; Auvynet *et al.*, 2013). Furthermore, and of particular relevance to this current study, exogenous Gal-1 has been shown to induce apoptosis in human neutrophils from synovial fluid of RA patients (Cedeno-Laurent *et al.*, 2010). *In vivo* (in the carrageenan-induced paw oedema model) Gal-1 was shown to inhibit neutrophil recruitment to the inflamed paw (Iqbal *et al.*, 2011) and trafficking of neutrophils is enhanced in Gal-1 KO mice (Cooper *et al.*, 2008). Gal-1 has been reported to have a pro-inflammatory role in the neutrophil respiratory burst, causing release of NADPH oxidase (Almkvist *et al.*, 2002).

Importantly, amongst current findings in the literature there is increasing evidence of potential pro-resolving actions for Gal-1. Several studies have demonstrated that expression of Gal-1 peaks at the height of the inflammatory response (Ilarregui *et al.*, 2009; Iqbal *et al.*, 2011; Starossom *et al.*, 2012). Moreover, Gal-1 was identified in resolving exudates and was down-regulated by the resolution-toxic anaesthetic

lidocaine (Chiang *et al.*, 2008). Differentiation of dendritic cells in a Gal-1 rich environment leads to enhanced regulatory functions and suppression of autoimmune disease progression thus indicating that Gal-1 acts in linking the innate and adaptive immune systems (Ilarregui *et al.*, 2009). Indeed, the pro-resolving potential of Gal-1 extends to cells of the innate immune system, where Gal-1 has been shown to induce 12/15-lipoxygenase expression and favour conversion to a pro-resolving phenotype in macrophages (Rostoker *et al.*, 2013) and induce phosphatidylserine exposure on the surface of neutrophils (Stowell *et al.*, 2007), thus suggesting Gal-1 has a pro-resolving role in macrophage efferocytosis of neutrophils.

A summary of the various roles of Galectin-1 on both innate and adaptive immune cells during inflammation and resolution can be seen in figure 4.

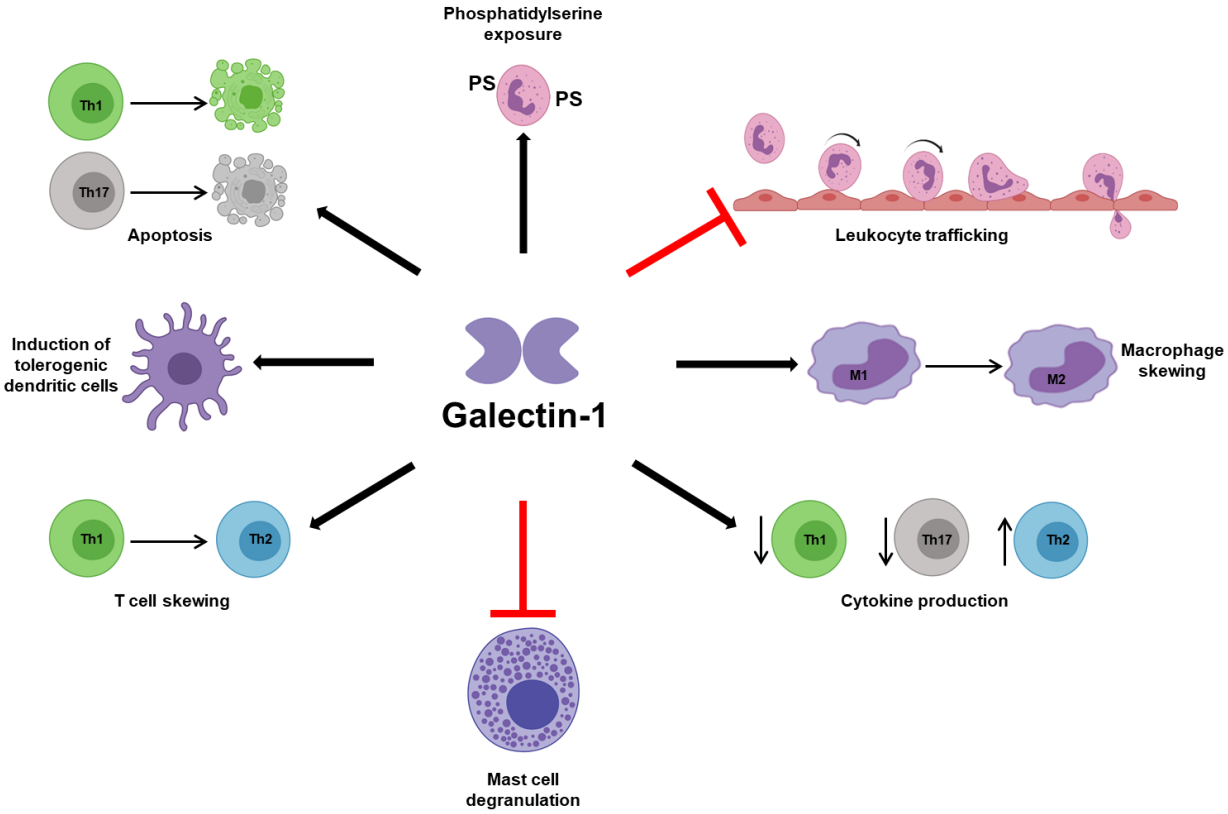



**Figure 4. Roles of Gal-1 in Inflammation and Resolution**
Galectin-1 (Gal-1) exerts anti-inflammatory and/or pro-resolving properties in inflammation. Anti-inflammatory actions include inhibitory effects on leukocyte trafficking and mast cell degranulation, as well as promotion of T cell apoptosis. Pro-resolving actions include instructing the adaptive immune response via T cell skewing and cytokine production as well as induction of tolerogenic dendritic cells. For cells of the innate immune system the pro-resolving actions of Gal-1 include increasing phosphatidylserine (PS) exposure on the surface of neutrophils and promotion of a pro-resolving (M2) phenotype in macrophages. [Created using BioRender illustrating tool.]

### *1.4.3. Gal-1 and Neutrophil Biology*

Few studies have investigated the effects of Gal-1 in the context of acute inflammation with new learnings on its interactions with neutrophils seldom reported.

It is well established that in the inflammatory response neutrophils are the primary leukocyte subset mobilised and that the initial step in this process is the establishment of leukocyte-endothelial interactions. Evidence in the literature has shown that Gal-1 is produced by the endothelium during inflammation (Baum, Seilhamer, *et al.*, 1995) a finding that first suggested that the protein may interact with neutrophils and play a role in their trafficking. To explore this potential relationship studies have been performed in our lab utilising both *in vitro* and *in vivo* methods with findings indicating that both exogenous and endogenous Gal-1 have an inhibitory effect on neutrophil trafficking. Furthermore, some of these earlier studies revealed Gal-1 inhibited specific neutrophil behaviours, including chemotaxis and trans-endothelial migration (La *et al.*, 2003).

An *in vitro* flow chamber system was utilised to test the effects of Gal-1, where neutrophils were flowed over human umbilical vein endothelial cells (HUVEC) stimulated with TNF-α, to resemble physiological events occurring in the inflamed microcirculation. Neutrophils pre-incubated with hrGal-1 prior to flow demonstrated that exogenous Gal-1 was able to significantly decrease the capture, rolling and adhesion of neutrophils onto the activated endothelial cells (Cooper *et al.*, 2008). Knockdown of endothelial Gal-1, using small interfering RNA, resulted in significantly increased neutrophil capture and rolling, further substantiating its role in neutrophil recruitment (Cooper *et al.*, 2008). Gal-1 inhibition of neutrophil trafficking has also

been shown *in vivo*. Increased neutrophil adhesion and emigration was seen in the inflamed cremasteric microcirculation of Gal-1 KO mice compared to WT by intravital microscopy (Cooper *et al.*, 2008). In a murine model of acute inflammation Gal-1 administration reduced neutrophil influx and oedema formation in a model of carrageenan induced paw oedema (Iqbal *et al.*, 2011) and inhibited interleukin-1β-induced PMN recruitment into the mouse peritoneal cavity (La *et al.*, 2003) demonstrating efficacy of the recombinant protein *in vivo*.

In addition to inhibiting neutrophil trafficking hrGal-1 also modulates adhesion molecule expression on the surface of activated neutrophils; addition of Gal-1 inhibited the decrease in P-selectin glycoprotein ligand 1 (PSGL-1) expression and concomitant increase in CD11b expression on platelet activating factor (PAF)-stimulated neutrophils (Cooper *et al.*, 2008).

Along with modulating surface expression of members of the integrin family that serve as markers of neutrophil activation, hrGal-1 also possesses the ability to induce the exposure of phosphatidylserine (PS) on the surface of neutrophils (Stowell *et al.*, 2009), an action typically associated with initiation of apoptosis (Denecker *et al.*, 2000). In addition to PS exposure, neutrophil apoptosis classically encompasses intracellular processes including mitochondrial dysfunction (resulting in depolarisation of the mitochondrial transmembrane potential) (Ly *et al.*, 2003), activation of caspases (proteases critical in the execution of apoptosis) (Shi, 2004) and DNA fragmentation. Intriguingly, these intracellular apoptotic programmes were not stimulated by hrGal-1 in the study by Stowell et al., thus suggesting that hrGal-1 induces PS exposure on neutrophils in the absence of initiation of apoptosis. Observations that neutrophil PS

exposure brought about by cell interaction with hrGal-1 is reversible when hrGal-1 is removed from the cell surface further contributes to comprehensive evidence that cell viability remains unaltered despite membrane changes of this manner usually being attributed to neutrophil apoptosis (Stowell *et al.*, 2009).

Furthermore, hrGal-1 induced exposure of PS on the surface of neutrophils is conditional of both the form of the protein and activation status of the cell. In this manner the dimeric form of the protein is required for successful signalling and the monomeric form of hrGal-1 is not able to induce PS exposure (Dias-Baruffi *et al.*, 2003). An additional study has also suggested that hrGal-1 induced surface exposure of PS does not occur on resting cells and is exclusive to neutrophils with an activated phenotype (Stowell *et al.*, 2007).

It is well established that Gal-1 readily binds to the surface of neutrophils (Dias-Baruffi *et al.*, 2003; La *et al.*, 2003; Cooper *et al.*, 2008). Current literature suggests that Gal-1 binding may be modulated in response to stimulation. It has been demonstrated that N-formyl-Methionyl-Leucyl-Phenylalanine (fMLP) stimulated neutrophils exhibited increased Gal-1 binding (Dias-Baruffi *et al.*, 2003) however, stimulation with PAF had no notable effect (Cooper *et al.*, 2008). Interestingly, neutrophils from the synovial fluid of rheumatoid arthritis patients, which are typically activated following their prolonged exposed to numerous mediators within the inflammatory milieu, are susceptible to Gal-1 induced apoptosis (Cedeno-Laurent *et al.*, 2010). Rather strikingly, this finding suggests that the activated phenotype of these neutrophils may render them susceptible to Gal-1-induced apoptosis.

The ability of Gal-1 to bind to neutrophils has been demonstrated to be carbohydrate dependent with binding of hrGal-1 inhibited in the presence of thiodigalactoside (Cooper *et al.*, 2008). Similarly, the 'flip' of PS to the outer membrane of activated neutrophils exposed to dimeric hrGal-1 was abolished in the presence of lactose (Dias-Baruffi *et al.*, 2003), suggesting that hrGal-1 signalling is a carbohydrate dependent mechanism requiring the protein to bind to cell surface β-galactosides.

Collectively these findings suggest that Gal-1 is endowed with the ability to influence neutrophil behaviour including neutrophil recruitment, clearance and survival, yet the mechanisms behind these functions remains to be fully elucidated. The specific behavioural features of Gal-1 have previously been shown to correspond with its location (Baum, Pang, *et al.*, 1995), nonetheless the involvement of Gal-1 and its influences on neutrophils and the mechanisms responsible for their clearance still remains poorly understood. As such these aspects will be investigated as part of this thesis, with emphasis on their potential involvement in inflammatory arthritis.

### *1.4.4. Gal-1 in Rheumatoid Arthritis*

The presence of Gal-1, -3, -8 and -9 in inflamed tissues, as seen in RA, indicates a key role for these galectins within inflammatory diseases (Mendez-Huergo *et al.*, 2019). Furthermore, galectins are detectable in the serum of RA patients (Ohshima *et al.*, 2003; Mendez-Huergo *et al.*, 2019). Of these galectin members with roles in RA, Gal-3 has been demonstrated to be predominantly proinflammatory and arthritogenic (Forsman *et al.*, 2011), whereas Gal-1, Gal-8 and Gal-9 are considered anti-inflammatory and protective (Rabinovich, Daly, *et al.*, 1999; Sebban *et al.*, 2007; Seki *et al.*, 2007; Iqbal *et al.*, 2013), when investigated *in vivo* using mouse models of RA.

The anti-inflammatory role of Gal-1 in arthritis was first reported by Rabinovich *et al.* in 1999. Utilising a T cell dependent model of RA they showed that therapeutic administration of either syngeneic fibroblasts engineered to secrete Gal-1 or exogenous recombinant Gal-1 suppressed arthritis by enhancing T cell susceptibility to antigen-induced apoptosis and reducing the anti-collagen antibody levels (Rabinovich, Daly, *et al.*, 1999). In the same study, exogenous Gal-1 was also shown to promote a shift in immune balance, by skewing the cytokine profile away from a type 1 T helper (Th1) cell mediated response and towards a type 2 T helper (Th2) cell humoral response. In line with these findings, the absence of endogenous Gal-1 resulted in increased susceptibility to arthritis with earlier onset of disease and increased severity, as well as elevated anti-collagen antibody levels (Iqbal *et al.*, 2013). Taken together these findings clearly demonstrate the inhibitory function of Gal-1 in the development and progression of arthritis.

Additional *in vitro* studies have further elaborated on the effects of Gal-1 on T cells with an arthritogenic phenotype, showing that Gal-1 inhibited the adhesion of IL-2 stimulated T cells to the extracellular matrix (ECM) and that this was due to blocking the re-organization of actin in activated T cells (Rabinovich, Ariel, *et al.*, 1999). The production of TNF-α and interferon-gamma (IFN-γ) by IL-2-activated T cells was also reduced by Gal-1, providing further evidence of its anti-inflammatory effect on arthritogenic T cells (Rabinovich, Ariel, *et al.*, 1999). Gal-1 also has a modulatory effect on trafficking in the inflammatory setting, a finding relevant to the augmented recruitment to the inflamed joint in RA. To this effect, exogenous Gal-1 reduced T cell trafficking along the activated endothelium whereas in the absence of the expression

of endogenous Gal-1 on activated endothelial cells T-cell adhesion was increased, thus demonstrating that Gal-1 is a negative regulator of T-cell recruitment (Norling *et al.*, 2008). These *in vitro* studies provide further evidence of the potential anti-inflammatory actions of Gal-1 in RA.

Aside from the effects of Gal-1 on T cells in RA very little work has been conducted on the role of the protein on other immune cells involved in the development and/or progression of the disease. Studies focused on the potential interplay between Gal-1 and the innate immune cells contributing to RA are especially limited. Findings by Rostoker *et al.* suggesting that Gal-1 promotes conversion of inflammatory macrophages to a pro-resolving phenotype that are quicker to reach efferocytic satiation (Rostoker et al., 2013), may have important translational relevance to macrophages present in the inflamed joint. However, this study was not performed on synovial macrophages and thus phenotypic differences may occur (Udalova *et al.*, 2016; Culemann *et al.*, 2019). As mentioned, Gal-1 can induce apoptosis of synovial fluid neutrophils from patients with RA (Cedeno-Laurent *et al.*, 2010). In the same study the granulocytes present in inflammatory synovial fluids from patients with RA were shown to express Gal-1 receptors. These results demonstrate that not only T cells but neutrophils from within the RA joint express Gal-1 receptors and their engagement with Gal-1 triggers apoptosis.

Despite low basal levels, Gal-1 expression is increased in response to inflammation with levels markedly increased in the sera from RA patients (Mendez-Huergo *et al.*, 2019). Furthermore, RA patients with moderate-high disease had high levels of Gal-1 whereas patients in remission or with low disease had comparatively lower levels (Vilar

*et al.*, 2019), positively correlating with disease activity thus suggesting its potential as a biomarker in RA. In contrast, Gal-1 levels are significantly reduced in the synovial fluid from patients with RA, which could be due to presence of Gal-1 autoantibodies (Xibillé-Friedmann *et al.*, 2013). It has been proposed that elevated Gal-1 autoantibodies within the synovial fluid of RA patients limits the amount of Gal-1 and thus prevents its anti-inflammatory actions within the inflamed joint.

## *1.5. Hypothesis and Research Questions*

Inflammation can often be neutrophil driven whereby they are the dominant leukocyte in the infiltrate. In such cases their extended life-span (Weinmann *et al.*, 2007) coupled with their cytotoxic potential prevents the successful shift from pro-inflammatory to pro-resolving. Thus, greater understanding of mediators, such as Gal-1, that demonstrate the potential to limit neutrophil recruitment and/or increase neutrophil apoptosis and clearance is required to expand on current knowledge of inflammatory disease pathogenesis.

### *1.5.1. Hypothesis*

This project challenges the hypothesis that the anti-inflammatory properties of Gal-1 are mediated through its ability to inhibit neutrophil recruitment and potentiate neutrophil clearance.

### *1.5.2. Research Questions*

To address the hypothesis there are three research questions that this project aims to answer:

1. Is Gal-1 protective in a neutrophil-driven model of inflammatory arthritis?
2. Can Gal-1 regulate neutrophil survival?
3. Does Gal-1 modulate macrophage phenotype and/or efferocytosis?

# Chapter 2: Materials and Methods

## *2.1. Materials*

### *2.1.1. Galectin-1*

Recombinant Human Galectin-1 was provided by GalPharma (Takamatsu, Kagawa, Japan). The recombinant protein is a cysteine-less mutant of WT Gal-1 with all cysteine residues substituted with serine residues (Nishi *et al.*, 2008). The cysteine residues in Gal-1 are particularly susceptible to oxidation, which renders the protein inactive. The cysteine-less mutant from of Gal-1 is resistant to oxidation whilst retaining all the known activities of WT Gal-1.

### *2.1.2. Leukocyte Isolation*

21-gauge butterfly needle (Hospira, Pfizer). 60ml syringe (Terumo). Dulbecco's phosphate buffered saline (DPBS) with ($^{+/+}$) or without ($^{-/-}$) calcium and magnesium, Histopaque 1077, Histopaque 1119, Sodium chloride (NaCl) and Sodium citrate (Sigma-Aldrich). Roswell Park Memorial Institute (RPMI) – 1640 Medium (Phenol Red free, without L-glutamine, with sodium bicarbonate) (Gibco, Thermo Fisher Scientific). Turk's solution prepared in-house (with 3% Acetic Acid (Sigma-Aldrich) and 0.01% Crystal Violet (Sigma-Aldrich)).

### *2.1.3. Cell Culture*

Accutase® (in $DPBS^{-/-}$ 0.5 mM Ethylenediaminetetraacetic acid (EDTA) with phenol red), Bovine Serum Albumin (BSA), L-glutamine (200mM) solution, Lipopolysaccharide (LPS) from *Escherichia coli* (*E. coli*) 0111:B4 and Penicillin-Streptomycin (P/S) (10,000 units penicillin and 10 mg streptomycin/ml) solution (Sigma-Aldrich). Amphotericin B (Fungizone) and Foetal Bovine Serum (FBS) (Gibco, Thermo Fisher Scientific). Mouse Recombinant Interleukin-4 (IL-4) (eBioscience). Human Recombinant Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), Human Recombinant Macrophage Colony-Stimulating Factor (hrM-CSF), Mouse Recombinant Macrophage Colony-Stimulating Factor (mM-CSF) and Mouse Recombinant Interferon-γ (IFN-γ) (PeproTech). 1.1ml Z-Gel Micro tubes (Sarstedt). RPMI – 1640 Medium (as listed above).

### *2.1.4. Fluorescent Labelling and Flow Cytometry*

α-mouse Fcγ block (clone 2.4G2, generated in-house, the hybridoma was grown and the supernatant harvested, contaminating cells and debris were removed by centrifugation). α-human Fcγ block (γ-Globulins from human blood, (Sigma-Aldrich)). Anhydrous Dimethyl Sulfoxide (DMSO) (Life Technologies, Thermo Fisher Scientific). Annexin V Binding Buffer (BD Pharmingen, BD Biosciences). BODIPY® FL N-(2-Aminoethyl) Maleimide, 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) and UltraComp ebeads (Invitrogen, Thermo Fisher Scientific). Carboxyfluorescein Succinimidyl Ester (CFSE), Fixation Buffer, Permeabilization Buffer and Fixation/Permeabilization diluent (eBioscience, Thermo Fisher Scientific). Paraformaldehyde (PFA) powder (Sigma-

Aldrich). BSA and DPBS$^{-/-}$ (as listed above). FACS buffer prepared in-house (DPBS$^{-/-}$ with 0.02% BSA).

**Table 3. Antibodies used for flow cytometry.**

| Marker | Fluorochrome | Host/Reagent | Isotype | Clone | Supplier | Conc. | Working Dilution |
|---|---|---|---|---|---|---|---|
| 7/4 | FITC | Rat α-Mouse | IgG2a | 7/4 | Abcam | 0.1mg/ml | 1:400 |
| AnnexinA1 | N/A | Mouse α-Human | IgG1 | 1B | Generated in house | 1mg/ml | 1:100 |
| AnnexinV | FITC | α-All Species | N/A | N/A | BD Pharmingen | - | 1:50 |
| AnnexinV | Pacific Blue | α-All Species | N/A | N/A | Biolegend | - | 1:50 |
| CD11b | BV785 | Rat α-Mouse | IgG2b κ | M1/70 | Biolegend | 50µg/ml | 1:400 |
| CD11b | Pe-Cy7 | Rat α-Mouse/Human | IgG2b κ | M1/70 | Biolegend | 0.2mg/ml | 1:1000 |
| CD11b | FITC | Rat α-Mouse/Human | IgG2b κ | M1/70 | Biolegend | 0.5mg/ml | 1:1000 |
| CD16 | PE | Mouse α-Human | IgG1 κ | 3G8 | BD Pharmingen | - | 1:50 |
| CD206 | APC | Mouse α-Human | IgG1 κ | 19.2 | eBioscience | 12µg/ml | 1:100 |
| CD206 | BV605 | Rat α-Mouse | IgG2a κ | C068C2 | Biolegend | 100µg/ml | 1:100 |
| CD36 | AF488 | Armenian Hamster α-Mouse | IgG | HM36 | Biolegend | 0.5 mg/ml | 1:100 |
| CD3e | PE-Cy5 | Armenian Hamster α-Mouse | IgG | 145-2C11 | EBioscience | 0.2mg/ml | 1:100 |
| CD43 | FITC | Mouse α-Human | IgG1 κ | 1G10 | BD Pharmingen | - | 1:42 |
| CD44 | N/A | Rat α-Human | IgG2b κ | IM7 | eBioscience | 0.5mg/ml | 1:200 |
| CD45 | PerCP | Rat α-Mouse | IgG2b κ | 30-F11 | Biolegend | 0.2mg/ml | 1:200 |
| CD45 | eFluor450 | Rat α-Mouse | IgG2b κ | 30-F11 | eBioscience | 0.2mg/ml | 1:200 |
| CD45 | PE | Mouse α-Human | IgG1a κ | 2D1 | eBioscience | 50µg/ml | 1:100 |
| CD64 | APC | Mouse α-Mouse | IgG1 κ | X54-5/7.1 | Biolegend | 0.2mg/ml | 1:100 |
| CD66b | PE | Mouse α-Human | IgM κ | G10F5 | Biolegend | 12.5µg/ml | 1:100 |
| CD86 | BV421 | Rat α-Mouse | IgG2a κ | GL-1 | Biolegend | 0.2mg/ml | 1:100 |
| CD86 | PE | Mouse α-Human | IgG2b κ | IT2.2 | Biolegend | 0.2mg/ml | 1:100 |
| CD86 | PE | Rat α-Mouse | IgG2b κ | PO3 | Biolegend | 0.2mg/ml | 1:200 |
| F4/80 | BV421 | Rat α-Mouse | IgG2a κ | BM8 | Biolegend | 0.05mg/ml | 1:200 |
| F4/80 | BV650 | Rat α-Mouse | IgG2a κ | BM8 | Biolegend | 0.2mg/ml | 1:100 |
| F4/80 | APC | Rat α-Mouse | IgG2a κ | BM8 | eBioscience | 0.2mg/ml | 1:100 |
| Gal-1 | N/A | Goat α-Human | - | - | R&D Systems | 0.2mg/ml | 1:100 |
| Gal-1 | N/A | Goat α-Mouse | - | - | R&D Systems | 0.2mg/ml | 1:100 |
| Goat IgG | FITC | Swine α-Goat | - | - | Caltag Laboratories | - | 1:200 |
| Goat IgG | N/A | Rabbit α-Goat Biotinylated | - | - | Dako | 1.6mg/ml | 1:500 |
| Ly6C | PerCpCy5.5 | Rat α-Mouse | IgG2c k | HK1.4 | eBioscience | 0.2mg/ml | 1:100 |
| Ly6G | PE | Rat α-Mouse | IgG2a κ | 1A8 | BD Pharmingen | 0.2mg/ml | 1:200 |
| MerTK | APC | Rat α-Mouse | IgG2a κ | 2B10C42 | Biolegend | 0.2 mg/ml | 1:100 |
| MHC-II | AF700 | Rat α-Mouse | IgG2b κ | M5/114.15.2 | Biolegend | 0.5mg/ml | 1:400 |
| Mouse IgG | AF488 | Goat α-Mouse | - | - | Invitrogen | 2mg/ml | 1:400 |
| Propidium Iodide | PI | α-All Species | N/A | N/A | BD Pharmingen | 50 µg/ml | 1:50 |
| Rat IgG | AF647 | Chicken α-Rat | Igy | - | ThermoFisher | 2mg/ml | 1:500 |
| Siglec-F | APC | Rat α-Mouse | IgG1 | ES22-10D8 | Miltenyi Biotech | 30µg/ml | 1:200 |
| Streptavidin | BV605 | Streptavidin α-Biotin | - | - | Biolegend | 0.1mg/ml | 1:200 |
| Zombie Aqua | FVD Aqua | α-All Species | N/A | N/A | Biolegend | - | 1:200 |
| Zombie NIR | NIR | α-All Species | N/A | N/A | Biolegend | - | 1:400 |

All antibodies used at working dilution in 50µl per well FACS (Fluorescent Activated Cell Sorting) buffer (DPBS$^{-/-}$ + 0.02% BSA) unless otherwise specified.

### *2.1.5. In vivo and ex vivo Sample Processing*

0.3ml MicroFine syringe with 30-gauge needle, 0.5ml insulin syringe with 27-gauge needle, 1ml syringe and 5ml syringe (Terumo). 25-gauge needle (BD Microlance 3). DPBS tablets (Oxoid, Thermo Fisher Scientific). EDTA 0.5M solution and RNAlater® (Ambion, Thermo Fisher Scientific). Entellan® mounting medium and Eosin Y-solution (Merck Millipore). Formaldehyde Solution 37% (Fisher Scientific, Thermo Fisher Scientific). Heparin Sodium (Leo Pharma). Histo-Clear (Scientific Laboratory Supplies). Rapid-Chrome Kwik-Diff Staining System (Shandon, Thermo Fisher Scientific). Collagenase D (from *Clostridium histolyticum*), Deoxyribonuclease (DNAse) I (from Bovine pancreas), Ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA disodium salt), Mayer's Haematoxylin, Sodium Hydroxide Pellets, Sodium phosphate monobasic monohydrate, Sodium phosphate monobasic dihydrate and Zymosan A (from *Saccharomyces cerevisiae*) (Sigma-Aldrich). 1.1ml Z-Gel Micro tube and 1.3ml EDTA Micro tube (Sarstedt). K/BxN serum (generated as described in section 2.2.4.1 from an in-house breeding colony crossing KRN and NOD mice). BSA, $DPBS^{-/-}$, hrGal-1 and PFA (as listed above).

### *2.1.6. Genotyping*

Agarose (Bioline), 10 x Tris-acetate EDTA (TAE) (Roche, Sigma-Aldrich). GelRed® (Biotium). Neutralization Solution B, oIMR6918, oIMR6919 and oIMR8306 primers, REDExtract-N-Amp™ Tissue PCR Kit (containing Tissue Preparation Solution, Extraction Solution and REDExtract-N-Amp PCR Reaction Mix) (Sigma-Aldrich). Quick-Load® Purple 100bp DNA Ladder (New England Biolabs). RNAse free water (Ambion, Thermo Fisher Scientific).

*2.1.7. Western Blotting*

0.1 M 1,4-Dithiothreitol (DTT), NuPAGE® LDS Sample Buffer 4X, NuPAGE® 10% Bis-Tris 12 well Gel and NuPAGE® MOPS SDS Running Buffer (Invitrogen). 23-gauge needle (BD Microlance 3). XCell SureLock™ Mini-Cell unit (Thermo Fisher). Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific). Glycine (Fisher Scientific). Tris base, Tris-HCL and Tween20 (Sigma-Aldrich). Transfer tank (Bio-rad). Skimmed milk powder (Marvel). Immobilon forte western HRP substrate and Immobilon-P Transfer Membrane (Merck Millipore). Super RX-N Medical X-ray Film (Fujifilm). 1ml syringe, BSA and Sodium Chloride as previously listed.

**Table 4. Antibodies used for Western blotting.**

| Protein (Residue) | Host | Clone | Supplier | Concentration | Working Dilution | Buffer | Incubation |
|---|---|---|---|---|---|---|---|
| Annexin A1 | Mouse | 1B | Generated in house | 1mg/ml | 1:2000 | 5% Milk TBS-T | Overnight, 4°C, rocking |
| β-Actin | Mouse | AC-15 | Sigma-Aldrich | 2mg/ml | 1:10000 | 5% Milk TBS-T | 1 hour, RT, rocking |
| Phospho AKT (ser473) | Rabbit | D9E | Cell Signalling | - | 1:1000 | 5% BSA TBS-T | Overnight, 4°C, rocking |
| Phospho ERK 1/2 (Thr202/Tyr204) | Rabbit | D13.14.4E | Cell Signalling | - | 1:1000 | 5% BSA TBS-T | Overnight, 4°C, rocking |
| Phospho p38 (Thr180/Tyr182) | Rabbit | 3D7 | Cell Signalling | - | 1:1000 | 5% BSA TBS-T | Overnight, 4°C, rocking |
| Total AKT | Rabbit | Poly | Cell Signalling | - | 1:1000 | 5% BSA TBS-T | Overnight, 4°C, rocking |
| Total ERK | Rabbit | Poly | Cell Signalling | - | 1:1000 | 5% BSA TBS-T | Overnight, 4°C, rocking |
| Total p38 | Rabbit | Poly | Cell Signalling | - | 1:1000 | 5% BSA TBS-T | Overnight, 4°C, rocking |
| Mouse IgG | Goat | - | Dako | 1.0mg/ml | 1:1500 | 5% Milk TBS-T | 1 hour, RT, rocking |
| Rabbit IgG | Goat | - | Dako | 0.25mg/ml | 1:2000 | 5% Milk TBS-T | 1 hour, RT, rocking |

## *2.2. In vivo and ex vivo Methods*

### *2.2.1. Ethics and Regulations*

Experiments performed *in vivo* all adhered to the Home Office regulations (Scientific Procedures Act, 1986) and were additionally approved under the guidelines set down by the Ethical Committee for the Use of Animals, Barts and The London School of Medicine. Additionally, protocols under the supervision of the above were accomplished baring careful consideration to the principles set out by the National Centre for Replacement Refinement and Reduction of Animals in Research (NC3Rs).

### *2.2.2. Mice*

Male wildtype (WT) C57/BL6 mice, were purchased from Charles River (Kent, UK), Gal-1 knock out (KO) mice were from a colony bred and housed at the same facility.

The age of the mice varied from 6-14 weeks dependant on the nature of the experimental work and where required WT and KO mice were both age and sex matched. Mice were housed within individually ventilated cages (IVCs), a maximum of six mice per cage in a facility with a 12-hour light-dark cycle and ad libitum feeding of a standard laboratory chow diet and water.

The genotype of the mice from the Gal-1 KO colony was routinely checked by extracting DNA from tissue samples of tail clippings. Genotype specific primers were added, and the DNA section amplified by a Polymerase Chain Reaction (PCR) cycle

which was followed by gel electrophoresis to visualise the final products, as described in section 2.4.3.

### *2.2.3. Zymosan-induced Peritonitis*

Described as a simple experimental system for the study of inflammation (Cash *et al.*, 2009), zymosan induced peritonitis is most widely used in the assessment of short-term acute inflammation and importantly the subsequent resolution. The induction of an innate immune response in this model can be applied to investigate a variety of behaviours, including (but not limited to) stimulation of inflammatory mediator production, activation of complement and leukocyte recruitment as well as mechanisms of phagocytosis and efferocytosis (Aderem and Underhill, 1999).

The i.p. administration of zymosan produces a common recruitment profile of inflammatory cells into the peritoneal cavity. Neutrophils are the first leukocyte to infiltrate the cavity, with their peak in number determining the peak of the inflammatory profile (Newson *et al.*, 2014). Monocyte influx follows, whereby the recruited inflammatory monocytes mature into macrophages and neutrophil numbers decline respectively. The capacity of this model to quantify leukocyte recruitment has determined it suitable for many investigations. Thus, its applications now far extend its initial use to monitor the routine response seen in acute inflammation that is followed by self-resolution. The method is now frequently applied to the study of more novel factors and is suitable for considering endogenous anti-inflammatory pathways, the effect of mediator ablation, potential drugs and therapeutic strategies. The actions/responses attributed to both the endogenous and exogenous effects of individual cells, pathways and mediators can be assessed in this model. Specifically,

application to a knockout host can be used to examine the effects of endogenous mediators, whereas additional treatment, either prophylactically or therapeutically, can be used to consider the outcomes of exogenous agents.

Much of the literature indicates that the inflammatory response to the typical acute dose of 1mg will resolve within around 72 hours post challenge although some resolving mediators remain elevated until at least 96h post zymosan (Bannenberg *et al.*, 2005; Damazo *et al.*, 2006). The time point to resolution varies dependent on dose, with studies administering a lower 10µg dose evidencing 48 hours as the time frame to complete resolution (Cash *et al.*, 2009). In this manner the 10mg high dose delayed resolving model will not typically be resolved until day 9 post challenge with some mediators still remaining increased at day 13 post zymosan (Fujieda *et al.*, 2013; Newson *et al.*, 2014).

In this current study a model of sterile peritonitis using zymosan as an inflammatory agonist to elicit an acute inflammatory response within the peritoneal cavity was used to assess the effect of Gal-1 on leukocyte recruitment and clearance. Application here utilised both the low (1mg) dose and high (10mg) dose models of zymosan peritonitis to respectively initiate an acute and delayed self-resolving inflammatory response.

#### *2.2.3.1. Acute Resolving Model*

Peritonitis was performed with Gal-1 KO mice and WT counterparts or with WT mice administered hrGal-1 (10µg) to assess the effects of both endogenous and exogenous Gal-1. Mice aged 6-8 weeks were administered 1mg of zymosan in 500µl $DPBS^{-/-}$ by i.p. injection at the 0h time point. Mice treated with hrGal-1 were given a dose of 10µg in 200µl $DPBS^{-/-}$ i.p. at either 2h post zymosan (during the initiation phase of response) or at 8h post zymosan (during the peak phase of response). A 0.5ml insulin syringe with 27-gauge needle was used to perform the above i.p. injections.

Mice were sacrificed at various time points (2, 6, 8, 24 or 48h) post zymosan, as depicted in figure 5A-C. Prior to its separation from the peritoneal cavity the skin above the peritoneum was sprayed with 70% ethanol and an incision made allowing for it to be pulled back and peritoneal lavage to be subsequently performed. A 25-gauge needle head attached to a 5ml syringe was used to administer 5ml ice cold $DPBS^{-/-}$ containing 2mM EDTA into the peritoneal cavity. The peritoneal cavity was gently massaged to dissociate attached cells and peritoneal lavage fluid was retrieved with a plastic pasteur pipette inserted into the cavity through a small incision made in the peritoneum. Exudate obtained was immediately transferred into 15ml falcon tubes and kept on ice.

### *2.2.3.2. Delayed Resolving Model*

Peritonitis was performed with mice aged 6-8 weeks by administering 10mg zymosan in 500µl $DPBS^{-/-}$ by i.p injection at the 0h time point as described above. WT mice were administered hrGal-1 (10µg) or vehicle (200µl $DPBS^{+/+}$) only control by daily i.p. injection from 24h post-zymosan (during the peak phase of response). The high dose of inflammogen used to initiate the inflammatory response in this model results in an extended period of recruitment and infiltration to the peritoneal cavity in an attempt to clear the insult. The influx is continuous for several days, as opposed to only hours in the acute (1mg) model and although the inflammation remains self-resolving resolution is considerably delayed in comparison.

Mice were sacrificed at various time points (24, 72 and 120h) post zymosan, as depicted in figure 5D. Peritoneal lavage was performed as in 2.2.3.1.

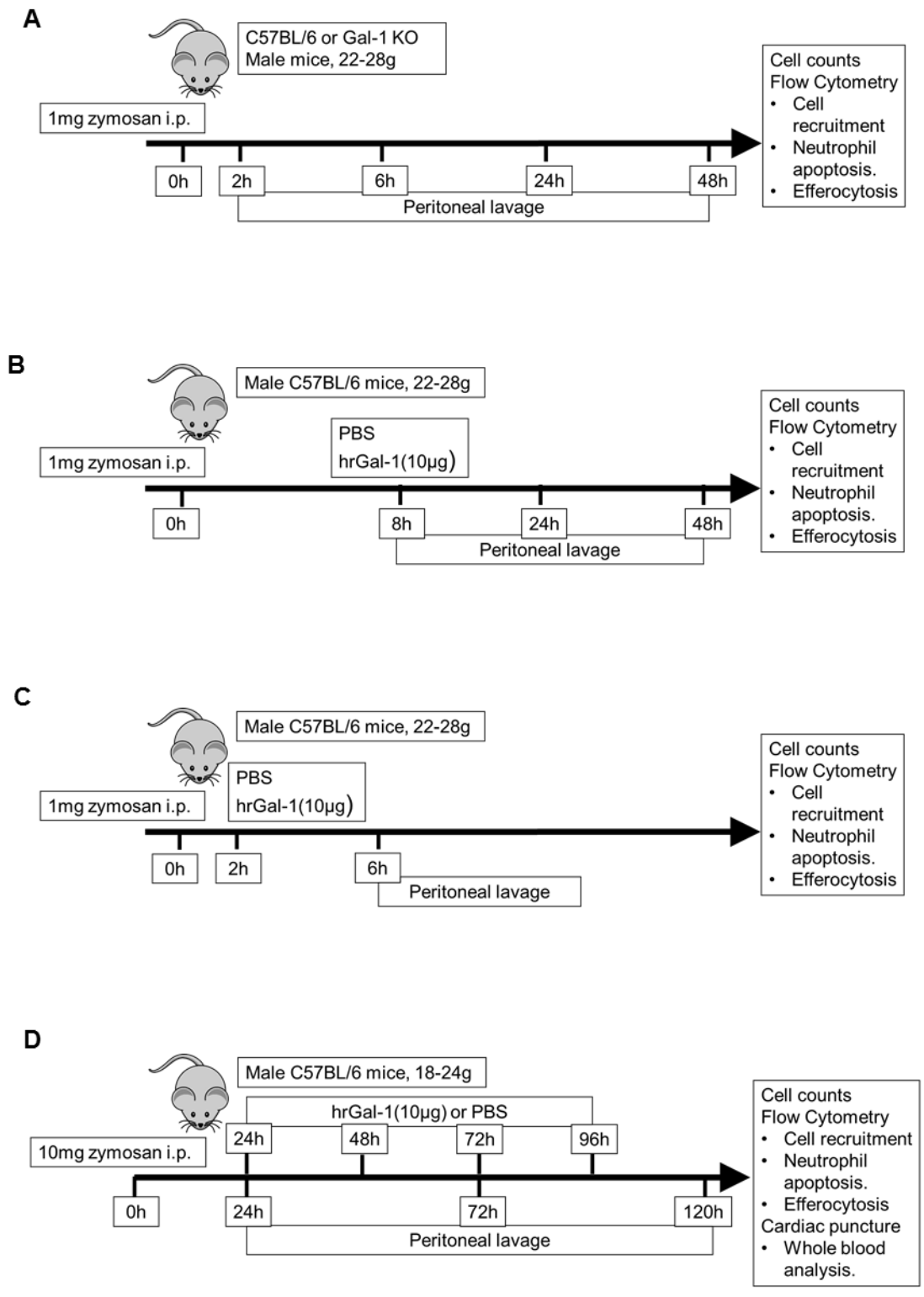
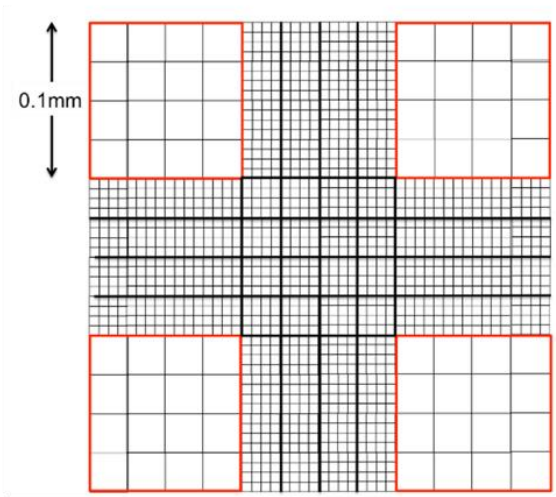



**Figure 5. Schematic time course for zymosan induced peritonitis model.**
(**A**) The timeline for endogenous Gal-1 protocols with peritoneal lavage performed at 2h, 6h, 24h, 48h. (**B**) The timeline for exogenous hrGal-1 protocols with treatment given at 8h and peritoneal lavage performed at 8h, 24h, 48h. (**C**) The timeline for exogenous hrGal-1 protocols with treatment given at 2h and peritoneal lavage performed at 6h. (**D**) The timeline for exogenous hrGal-1 protocol with treatment given daily from 24h and peritoneal lavage performed at 24h, 72h and 120h. Cardiac punctures were performed and whole blood was profiled from 72h and 120h timepoints.

### *2.2.3.3. Total Cell Number*

Total cell numbers in the peritoneum were determined by cell counts using a Neubauer haemocytometer chamber. An Olympus BH-2 standard light microscope was used to visualise cells stained with Turk's Solution on a Neubauer counting chamber at x40 objective. Cells were counted in the larger four outer squares of the counting chamber, as indicated in figure 6. Total cell counts per mouse were calculated based on 5ml of lavage fluid within the peritoneal cavity.

**A**




**B**

$$\frac{\text{Total cells counted in all four corners}}{4} \times 10^4 \times \text{dilution factor} = \text{cells per ml}$$

**Figure 6. Cell counting using a Neubauer haemocytometer chamber.**
(**A**) Counting grid on which cells are counted in each of the larger 4 outer corner squares (each composed of a 4x4 grid) as indicated by the red squares. (**B**) Formula to calculate total number of cells per ml from the number of cells counted using the chamber.

#### *2.2.3.4. Cell Recruitment and Clearance*

The peritoneal exudate retrieved was then analysed by flow cytometry for inflammatory cell type, neutrophil apoptosis (*in vivo* and *ex vivo*) and efferocytosis. Prior to staining cells with surface antibodies, the peritoneal exudate was vortexed, and 200µl was plated into a 96-well U-bottomed plate. Cells were pelleted by centrifugation (30 secs @ 800g) and the supernatant was discarded. Fc block diluted 1:5 in FACS buffer (50µl per well) was added and cells were resuspended and incubated for 20 mins, on ice. After incubation addition of (100µl per well) FACS buffer (DPBS-/- with 0.02% BSA) was used to wash. Again, cells were pelleted by centrifugation and the supernatant discarded.

For identifying immune cells, a panel of (CD11b, CD45, F4/80, Ly6G, SiglecF and 7/4) fluorescently conjugated antibodies at working dilution (Table 3) were added, cells were resuspended, covered and incubated for 30 mins on ice. Cells were washed twice with FACS buffer, pelleted by centrifugation, and the supernatant discarded before finally being resuspended in FACS buffer (200µl), transferred into FACS tubes and fixed by the addition of 4% PFA solution (50µl). The 4% PFA solution was prepared from PFA powder in $DPBS^{-/-}$ as follows: $DPBS^{-/-}$ was heated to 60°C on a heated stirring plate, PFA powder (w/v), was added followed by NaOH (dropwise) to elevate the pH and facilitate dissolution, once dissolved additional $DPBS^{-/-}$ was added to bring the solution to the required total final volume and the pH adjusted to 6.9 by addition of HCl (dropwise).

Leukocyte (CD45+) subsets were identified using subtype-specific antibodies. Specifically populations of neutrophils (7/4+, Ly6G+) inflammatory monocytes (7/4+,

Ly6G-), macrophages (F4/80+) – including resolving macrophages (F4/80+, CD11b low) and mature macrophage (F4/80+, CD11b high) subsets and eosinophils (SiglecF+) were detected. Figure 7 shows the gating strategy used for this analysis. Firstly, doublet exclusion was performed using a forward scatter area (FSC-A) against forward scatter height (FSC-H) plot followed by a side scatter area (SSC-A) against side scatter width (SSC-W) plot. The single cell moieties were then used for subset specific leukocyte identification, which was quantified using CD45+ cells as the parent population and the subsequent percentage of positive cells by specific biomarker.

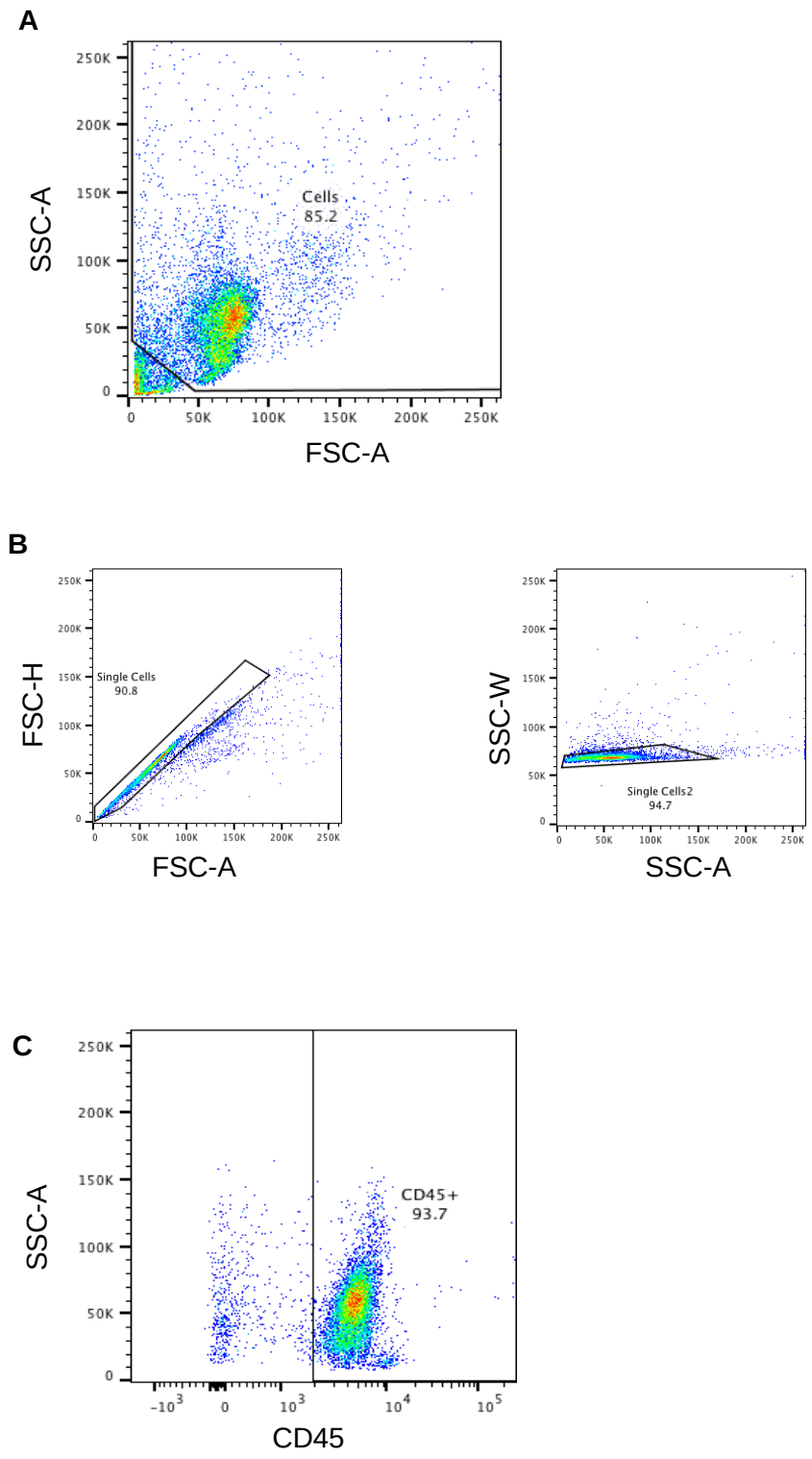



**Figure 7. Zymosan induced peritonitis leukocyte gating strategy.**
(A) Murine leukocytes collected from peritoneal lavage were displayed on a Forward Scatter Area (FSC-A) against Side Scatter Area (SSC-A) plot and gated on to exclude debris. (B) Single cell moieties were obtained from the population by first plotting FSC-A against Forward Scatter Height (FSC-H) to exclude doublet cells, followed by further doublets excluded by plotting this population on a SSC-A against Side Scatter Width (SSC-W) axis. (C) The population of singlets was plotted on an axis with the bandpass filter for the CD45 marker against SSC-A. This determined the leukocyte ($CD45^+$) population from other events obtained. The relative cell numbers for specific hematopoietic cell types were calculated using this population.

For *in vivo* neutrophil apoptosis peritoneal exudates were analysed immediately post collection. For *ex vivo* neutrophil apoptosis 1ml peritoneal exudate was spun (5 mins @ 400g, RT) in a microcentrifuge, supernatant was collected (and stored at -80°C) for cytokine analysis. Pelleted cells were resuspended in RPMI – 1640 Medium + 0.5% BSA (1ml) and plated into 12-well cell culture plates. Plates were incubated in a humidified chamber in 5% $CO_2$ at 37°C for 20 hours, after which 200µl cell suspension was plated into a 96-well U-bottomed plate. Cells were pelleted, Fc block added, and a wash performed (as described above).

In both *in vivo* and *ex vivo* analyses an antibody to Ly6G, a marker expressed on murine neutrophils, was added, cells were resuspended and covered and incubated (30 mins, on ice). Cells were washed twice, pelleted, and the supernatant discarded followed by the addition of AnnexinV and Zombie (NIR or Aqua) or Propidium Iodide (PI) in (50µl per well) AnnexinV binding buffer. Cells were resuspended, covered and incubated (15 mins, at room temperature (RT)). After incubation 150µl AnnexinV binding buffer was added to each well and well contents were transferred to FACS tubes. Cells were analysed immediately by flow cytometry.

Apoptosis of neutrophils was determined by first gating Ly6G positive cells, and then analysing the AnnexinV/Zombie or AnnexinV/PI staining by quadrant gating on the viable, early apoptotic, late apoptotic and necrotic populations. The gating strategy applied is shown in figure 8.

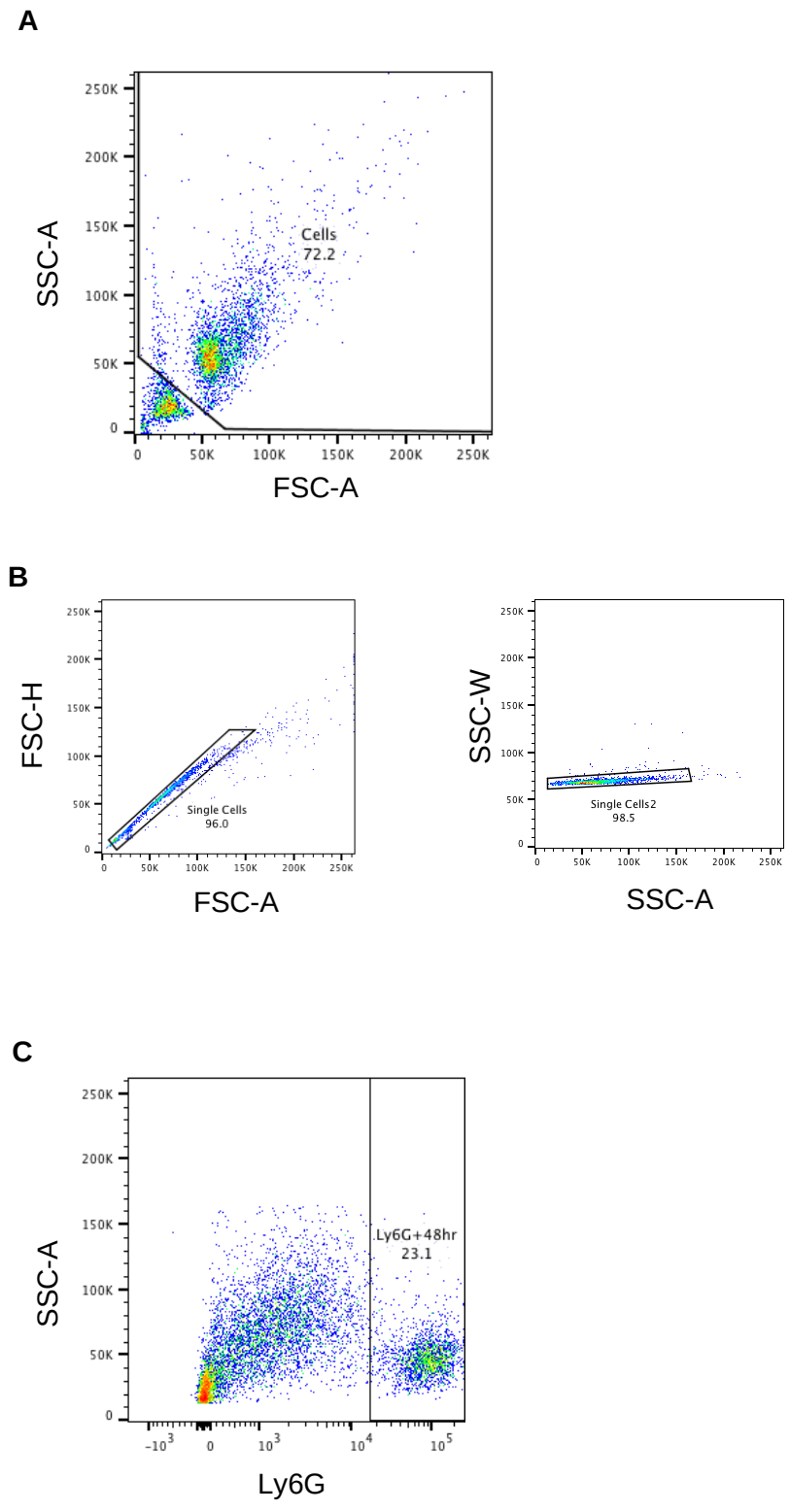



**Figure 8. Zymosan induced peritonitis neutrophil gating strategy.**
(A) Murine leukocytes collected from peritoneal lavage were displayed on a Forward Scatter Area (FSC-A) against Side Scatter Area (SSC-A) plot and gated on to exclude debris. (B) Single cell moieties were obtained from the population by first plotting FSC-A against Forward Scatter Height (FSC-H) to exclude doublet cells, followed by further doublet exclusion by plotting this population on a SSC-A against Side Scatter Width (SSC-W) axis. (C) The population of singlets was plotted on an axis with the bandpass filter for the Ly6G marker against SSC-A. This determined the neutrophil ($Ly6G^+$) population from other events obtained. The subsequent percentages of neutrophil cell state were calculated using this population.

For efferocytosis a standard protocol for fixation followed by permeabilisation of cells was used. Firstly, an antibody to surface F4/80, an antigen expressed at high levels on peritoneal macrophage populations, was added, cells were resuspended, covered and incubated (30 mins, on ice). Following incubation cells were washed, pelleted, and the supernatant discarded, twice. Cells were resuspended in fixation buffer (200µl), covered and incubated (30 mins, at RT). Cells were pelleted, and the supernatant discarded. Permeabilisation buffer (200µl) was added in place of FACS buffer and the wash cycle performed twice. Secondly anti-Ly6G diluted in permeabilisation buffer (50µl) was added and cells were incubated (30 mins, at RT). The wash cycle was then performed three times. Finally, the cells were resuspended in FACS buffer (200µl) and transferred into FACS tubes for analysis. Efferocytosis was determined by gating on the macrophage specific surface biomarker (F4/80) followed by assessing this population for cells positive for the neutrophil specific biomarker (Ly6G), with the gating strategy shown in figure 9.

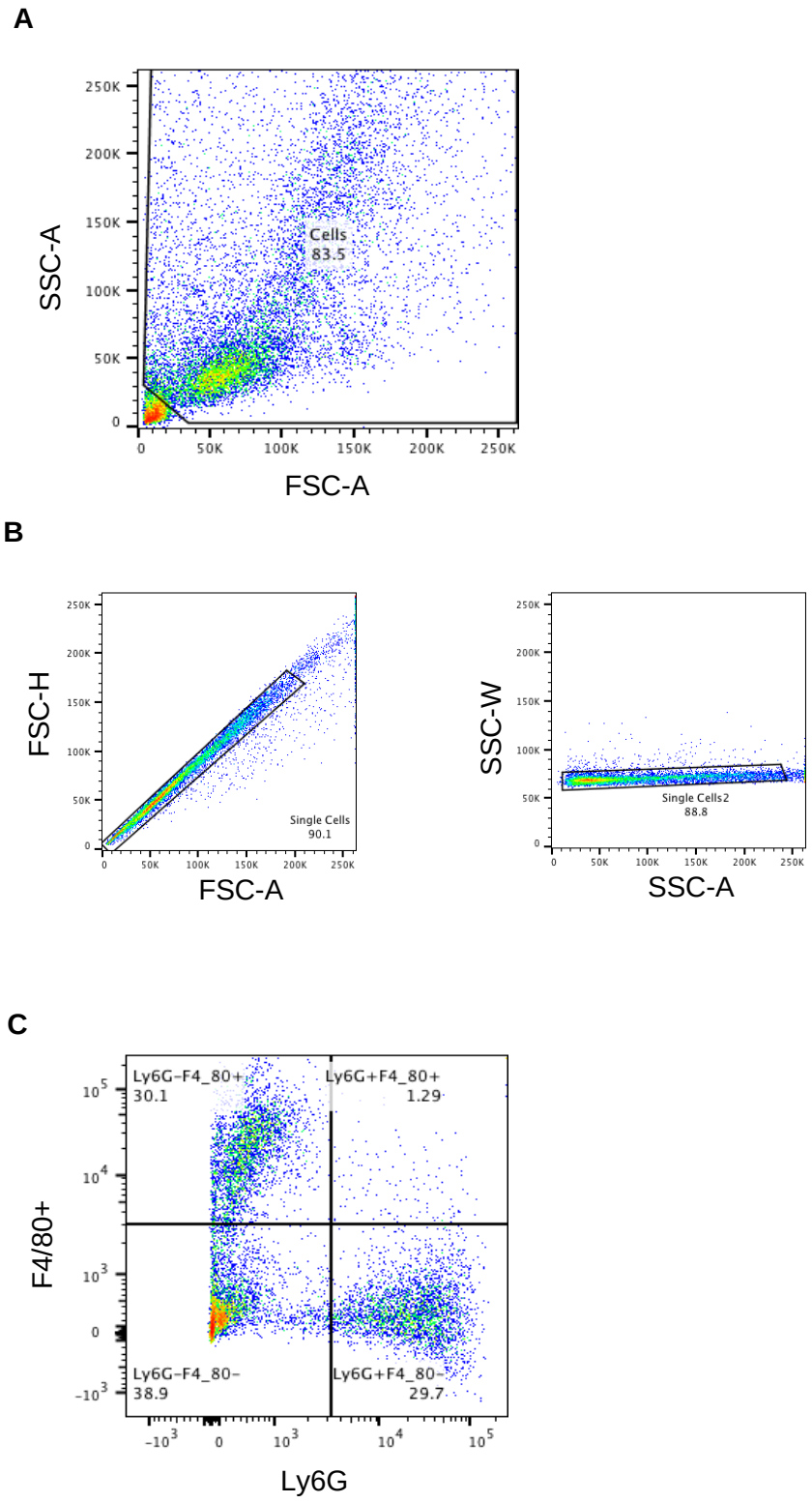



**Figure 9. Zymosan induced peritonitis efferocytosis gating strategy.**
(A) Murine leukocytes collected from peritoneal lavage were displayed on a Forward Scatter Area (FSC-A) against Side Scatter Area (SSC-A) plot and gated on to exclude debris. (B) Single cell moieties were obtained from the population by first plotting FSC-A against Forward Scatter Height (FSC-H) to exclude doublet cells, followed by further doublet exclusion by plotting this population on a SSC-A against Side Scatter Width (SSC-W) axis. (C) The population of singlets was plotted on an axis with the bandpass filter for the Ly6G antibody on the x-axis against F4/80 on the y-axis. This determined the neutrophil only ($Ly6G^+$), macrophage only ($F4/80^+$) populations as well as the population of double positive ($F4/80^+Ly6G^+$) macrophages i.e. those which had efferocytosed apoptotic neutrophils.

#### *2.2.3.5. Inflammatory Mediator Analysis*

Peritoneal exudate (1ml) was centrifuged (5 mins @ 400g, RT) and supernatant was collected and stored at -80°C. The supernatant samples from peritoneal lavages obtained 2h post zymosan were sent to Labospace (LaboSpace Ltd. Milan, Italy). Levels of analytes (see table 5) were measured using a custom designed mouse Luminex screening assay.

## Table 5: Inflammatory mediators analysed in peritoneal exudate.

| Cytokine | Class | Production | Target Cell (Interaction) | Functions | Detection Range (pg/ml) |
|---|---|---|---|---|---|
| GM-CSF | Cytokine (growth factor) | Macrophages, T cells, mast cells, NK cells, endothelial cells and fibroblasts. | Granulocytes and Macrophages. | Recruitment and alteration of receptors expressed on neutrophils. Activation of macrophages. | 5 - 2800 |
| IL-1β | Cytokine (pro-inflammatory) | Tissue macrophages, monocytes, fibroblasts and dendritic cells. Low levels by B lymphocytes, NK cells and epithelial cells. | Macrophages and endothelial cells. | Increases the expression of adhesion factors on endothelial cells to enable transmigration, promoting the recruitment and retention of macrophages. | 75 - 61555 |
| IL-6 | Cytokine (pro-inflammatory) | Secreted by T cells, macrophages and monocytes to stimulate immune response. | Various cells during acute phase of response. | Regulation of inflammatory chemokines and apoptotic events dictating the profile of leukocyte recruitment. | 10 - 7205 |
| KC (CXCL1) | Chemokine | Induced by inflammatory cytokines (IL-1, TNF, PGE2 and thrombin). | Neutrophils and endothelial cells. | Recruitment of neutrophils to inflammatory sites. | 10 - 8320 |
| MCP-1 (CCL2) | Chemokine | Primarily by monocytes, macrophage and dendritic cells. | Monocytes and endothelial cells. | Monocyte recruitment into sites of immune responses and cancer. | 215 - 51655 |
| MIP-1-α (CCL3) | Chemokine | Activated macrophages, dendritic cells, lymphocytes, and endothelial cells. | Macrophages, monocytes and neutrophils. | Recruitment and activation of neutrophils during the acute inflammatory response. | 0 - 200 |
| TNFα | Cytokine (pro-inflammatory) | Primarily by monocytes and macrophages. | Macrophages. | Cell adhesion molecule (CAM) and cytokine expression. Induces inflammation and apoptosis. | 0 - 805 |
| VEGF | Cytokine (growth factor) | Primarily monocytes and macrophage | Endothelial cells and monocytes. | Activation and recruitment of monocytes. Enhances blood vessel permeability. | 0 – 1020 |

### *2.2.3.6. Bone Marrow Derived Macrophage Skewing*

Peritonitis was elicited in Gal-1 KO and WT mice by administration of 1mg of zymosan (in 500µl $DPBS^{-/-}$) by i.p. injection at 0h and mice were sacrificed at 2h post zymosan. Following peritoneal lavage, the right hind limb of each mouse was collected into falcon tubes containing RPMI – 1640 + 2% FBS + 1% P/S and stored on ice.

Bone marrow cells were collected from the femur of WT and Gal-1 KO mice following 2h of inflammatory stimulation. Cells flushed from the bone were plated and differentiated into bone marrow derived macrophages (BMDMs) before being stimulated with macrophage skewing cytokines (Narayan *et al.*, 2017; Rhys *et al.*, 2018; Wang *et al.*, 2020), as displayed in figure 10. Stimulated cells were collected and stained with a panel of fluorescently conjugated antibodies to key macrophage biomarkers (MerTK, CD36, CD206, MHC-II and CD86). Samples were analysed by flow cytometry to determine if phenotypic differences existed between WT and Gal-1 KO macrophage.

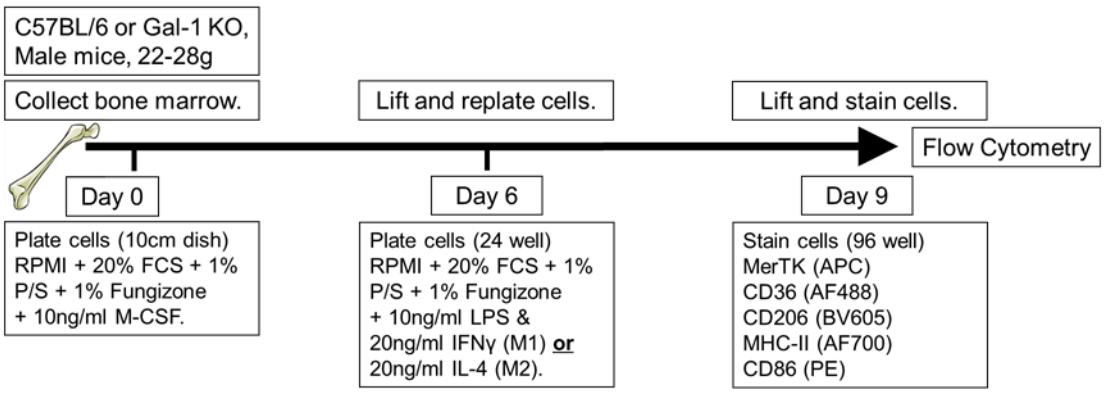



**Figure 10. Schematic time course for differentiation and skewing of bone marrow derived macrophages.** Mice (WT or Gal-1 KO) were sacrificed 2h post zymosan (1mg in 500µl PBS) and the right hind limb collected. Bone marrow was extracted from the femur, plated, differentiated to macrophage for 6 days and then stimulated with M1 (10ng/ml LPS + 20ng/ml IFN-γ) / M2 (20ng/ml IL-4) to polarise macrophages for 3 days. Macrophage phenotyping was performed by flow cytometry to detect surface presentation of MerTK, CD36, CD206, MHC-II and CD86.

To free the femur from the rest of the limb the muscle was first cut away from the lower extremity, next, whilst ensuring not to break the femur head, the acetabulum was carefully dislocated from the hip joint. Ligaments attaching the femur and tibia were then snipped with scissors and the bones detached from one another by a gentle twist and pull action. Any remaining muscle was cleaned off the femur using a piece of gauze.

To harvest the cells both ends were cut from the bone and the bevel of a 23-gauge needle attached to a 5ml syringe inserted into the bone marrow shaft. $DPBS^{-/-}$ + 1% P/S was slowly passed through the bone. Whilst flushing the needle head was used to gently scrape the inner surface of the bone to maximise cell recovery and flushing was performed until bone was clear. Bone marrow was passed through a 70µm cell strainer and collected in a 50ml falcon. The collected bone marrow was then transferred to a 15ml falcon and pelleted by centrifugation (10mins @ 425g).

Following centrifugation, the supernatant was discarded, and the pelleted cells resuspended in 10ml culture media (RPMI 1640 + 20% FBS + 1% P/S + 10ng/ml mM-CSF) and plated in 10cm cell culture plates. Plates were incubated at 37˚C, 5% $CO_2$ for 6 days allowing cells to differentiate into macrophages.

After 6 days of cell culture, BMDM were detached from the 10cm culture plate and replated into a 24-well cell culture plate for skewing. The media was aspirated, and the 10cm culture plates washed with $DPBS^{-/-}$. Macrophages were then incubated at 37°C in 5% $CO_2$ for 20 minutes with (3ml per plate) pre-warmed (37°C) accutase + 0.5mM EDTA. Following incubation to detach the cells the bottom of the plate was

gently swept with a cell scraper and a 3ml pasteur pipette was used to flush the accutase solution around the plate several times to loosen any macrophages that may have remained attached. Cells in solution were then collected in a (15ml) falcon tube and detachment of macrophages from the plate confirmed by microscopy. Any remaining cells were collected by flushing the plate several additional times with $DPBS^{-/-}$ (3ml). Falcon tubes were topped up to 15ml with $DPBS^{-/-}$ and macrophages were pelleted by centrifugation (10 mins @ 425g, RT).

Following centrifugation, macrophages were resuspended in 1ml media (RPMI – 1640 Medium + 20% FBS + 1% P/S). BMDM cultured from each mouse were then divided into 3 wells of a 24-well cell culture plate (300µl per well) to skew to M0, M1, and M2 phenotypes. For M0 no stimulant was added. For M1 (classical/pro-inflammatory) skewing macrophages were stimulated with 10ng/ml LPS and 20ng/ml IFN-γ whilst M2 (alternative / non-inflammatory) skewing of macrophages was performed by the addition of 20ng/ml IL-4. Macrophages were incubated in their stimulatory media at 37˚C in 5% $CO_2$ for 3 days.

After 3 days the cultured BMDM were detached as described above. Cells were resuspended in FACS buffer (200µl) and plated into a 96-well U-bottomed plate for staining for flow cytometric analysis. For detecting expression of macrophage phenotypic markers, a panel of (MerTK, CD36, CD206, MHC-II, CD86 and F4/80) fluorescently conjugated antibodies was used. Antibodies were added to wells at working dilution (see table 3) in (50µl) mouse Fc block (diluted 1:5 in FACS buffer), cells were resuspended in the solution, covered and incubated (30 mins, on ice). Cells were then washed twice with FACS buffer, pelleted by centrifugation, and the

supernatant discarded before finally being resuspended in FACS buffer (100µl), transferred into FACS tubes and fixed by the addition of 4% PFA solution (25µl). Samples were acquired (on an LSR Fortessa) and data was analysed by selecting the single cell populations, followed by F4/80+ and measuring the MFI for each of the antibody conjugated fluorochromes

#### *2.2.3.7. Peripheral Blood Profiling*

Peripheral blood was collected from mice and profiled across time in the delayed resolving model of peritonitis (as described in section 2.2.3.2). Briefly peritonitis was elicited by administering 10mg zymosan by i.p. injection at the 0h time point. WT mice were administered hrGal-1 (10µg) or vehicle (200µl $DPBS^{+/+}$) only control daily from 24h post-zymosan. At 72h and 120h post-zymosan blood was retrieved by cardiac puncture (as described in section 2.2.4.5.), withdrawn into a 1ml syringe and immediately transferred to 1.3ml EDTA Micro tubes.

Whole blood (50µl) was ran on the ProCyte DX Haematolgy Analyser (IDEXX) as per manufacturer's guidelines. The Analyser combines flow cytometry, optical fluorescence and laminar flow impedance to provide a comprehensive complete blood count including 27 parameters (see table 6).

## Table 6. ProCyte DX Haematolgy Analyser whole blood parameters.

| Parameter | Description |
|---|---|
| RBC | Total number of erythrocytes |
| HCT | Hematocrit value: erythrocyte ratio of total blood volume |
| HGB | Hemoglobin concentration |
| MCV | Mean erythrocyte volume in total sample |
| MCH | Mean haemoglobin volume per red clood cell (RBC) count |
| MCHC | Mean haemogobin concentration of erythocytes. |
| RDW | The degree of variation in size of the erythrocyte population |
| %RETIC | Reticulocyte percent |
| RETIC | Reticulocyte count |
| WBC | Total number of leukocytes |
| %NEU | Neutrophil percent |
| %LYM | Lymphocyte percent |
| %MONO | Monocyte percent |
| %EOS | Eosinophil percent |
| %BASO | Basophil percent |
| NEU | Neutrophil count |
| LYM | Lymphocyte count |
| MONO | Monocyte count |
| EOS | Eosinophil percent |
| BASO | Basophil percent |
| PLT | Total number of platelets |
| MPV | Mean platelet volume |
| PDW | Platelet distribution width; the degree of variation in the size of the platelet population |
| PCT | Plateletcrit value |

### *2.2.4. K/BxN Serum Transfer Arthritis*

An inflammatory polyarthritis was induced in mice by i.p. injection of K/BxN serum. Arthritogenic serum was produced in-house from KRN/NOD mice as previously described (Kouskoff *et al.*, 1996). The KRN/NOD colony was established from crossing KRN mice, which are heterozygous for the transgenic T cell receptor (TCR) KRN with NOD (non-obese diabetic; ShiLtJ) mice. Offspring (K/BxN) develop a spontaneous arthritis upon weaning and possess arthritogenic antibodies in their bloodstream (Kouskoff *et al.*, 1996; Christensen *et al.*, 2016). Consequently, serum isolated from these mice can induce disease in recipient animals. Arthritis was induced using a dosing strategy and volume of serum that elicits visible disease onset approximately three days post injection as determined in house.

#### *2.2.4.1. Model*

K/BxN serum transfer Arthritis was performed with Gal-1 KO mice and WT counterparts or with WT mice administered hrGal-1 (3µg) to assess the effects of both endogenous and exogenous Gal-1. Mice aged 10-12 weeks on a C57BL/6 background received two doses of K/BxN serum, the initial dose was given on day zero and the subsequent on day two, for each dose serum was diluted 1:1 with DPBS-/- and 200µl administered by i.p. injection.

#### *2.2.4.2. Disease Profile*

Mice were assessed daily for weight loss and oedema and clinically scored for arthritis. Paw oedema was measured by use of a digital plethysmometer, which involved immersing the mouse paw into the water where a sensor notes a pressure change,

outputting the volume displacement measured with 0.01 ml resolution. Both hind paws were measured, and a mean value calculated. Clinical scoring for each paw was measured on a semi-quantitative scale. The ankle/wrist, pad and digits of all four limbs were assessed and a score was given to each of these three factors. Features of the appendages that were visibly swollen received a score of one whilst those that appeared normal/healthy were scored with a zero. From these scores a total value (maximum 12) for each mouse was then calculated, the scoring system used is shown in figure 11.

To determine if endogenous Gal-1 has a role in this neutrophil driven model two separate experiments were performed. To obtain an idea of the full disease profile, where mice were monitored for 40 days and remission indices were calculated. Values indicated the time points when mice reached the maximum magnitude of arthritis (Tmax), when 50% of the maximum was reached ($T_{50}$) and the resolution interval (Ri) determined as the time from Tmax to $T_{50}$. A second experiment was performed for a duration of four days, a period determined based on the divergence previously seen between the two genotypes at this time and thus of interest to analyse the cellular infiltrate in both genotypes at that point.

To explore whether exogenous hrGal-1 would influence neutrophil driven arthritis. WT mice were treated with hrGal-1 (3µg) or vehicle (20µl $DPBS^{+/+}$) by intraplantar injection using a MicroFine syringe with 30-gauge needle. Mice were treated from day 3, when disease was established, and every alternate day thereafter before being sacrificed at the peak of inflammation (day 7) or during resolving inflammation (day 11).

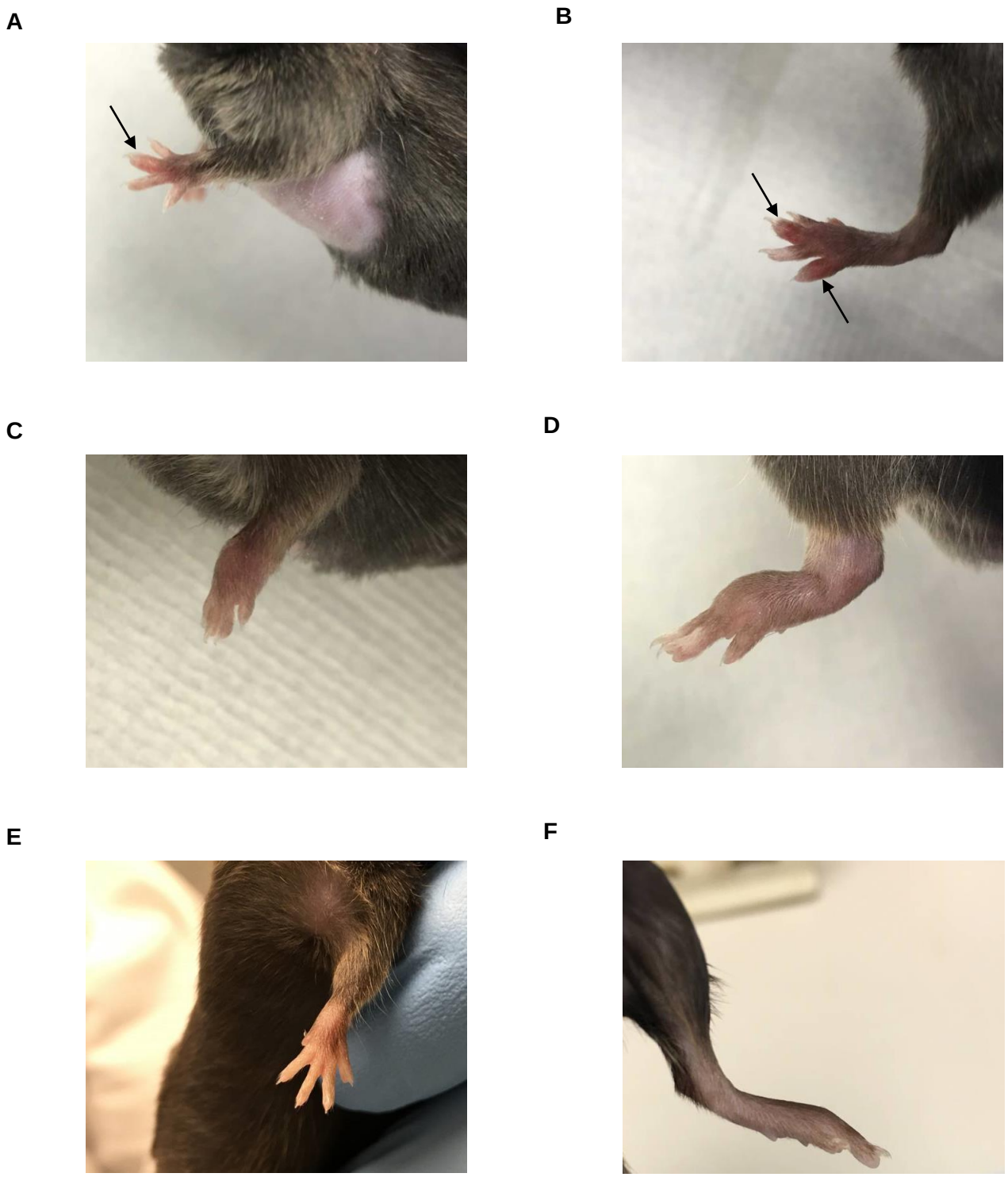



**Figure 11. K/BxN serum transfer induced arthritis clinical scoring.**
Digits, pads and ankle and wrist joints of mice given K/BxN serum were clinically assessed for swelling throughout the duration of the model. Digits of the upper (A) and lower (B) paws were given a score of one for inflamed digits, as indicated by black arrows. The wrist joint and pad of the upper limb (C) and ankle joint and foot pad of the lower limb (D) were also given a score of one for each independently swollen feature. All features were compared with the upper (E) and lower (F) limbs of naïve mice.

#### *2.2.4.3. Isolation of Cells from Arthritic Paws and Analysis by Flow Cytometry*

The effects of Gal-1 on cellular infiltrate into the paw was assessed for both the endogenous and exogenous protein due to the divergence in the role of the protein depending on its source. The role of endogenous Gal-1 on the cellular milieu within the inflamed paw was assessed at 4 days post K/BxN serum injection, a timepoint when inflammation is well established, but disease severity is still increasing. Exogenous Gal-1 was administered post disease establishment to assess its anti-inflammatory/pro-resolving potential. As such, the cellular content of inflamed paws was assessed at 11 days post K/BxN serum injection, when inflammation in the joint is typically beginning to cease and resolve. In both cases mice were sacrificed at the specified time points and hind paws were collected and placed in ice-cold RPMI – 1640 Medium prior to processing. Cells from the hind paws were isolated by performing a digestion protocol.

Firstly, the sock of hair around the ankle was removed followed by the rest of the skin. Dissection scissors were used to cut from the top of the ankle down to the tip of the middle digit, the layer of skin was then pulled back and removed by use of mechanical force and dissection tweezers. Further dissection of the paw was performed by carefully cutting between the digits for the length of the paw, ensuring not to break or damage the bones.

A digestion buffer was prepared by dissolving collagenase D (0.5ng/ml) and DNAse (40ng/ml) in RPMI – 1640 Medium, 15ml of this was then added to Erlenmeyer flasks. The dissected paws were each placed into their own flask along with a small metal

stirring bar and the flasks were covered. A first digestion cycle was then performed, whereby flasks were incubated at 37°C for 30 minutes on a magnetic stirrer set to a medium speed. The digestion solution was passed through a 70µm cell strainer and collected into 50ml falcon tubes containing 2ml FBS and kept on ice. A second digestion cycle, identical to the first, was performed with 15ml of fresh digestion buffer added to the dissected paws in the flasks. The digestion solution was again passed through a 70µm cell strainer and combined with the solution from the first round of digestion. The cell solution was spun (10 mins @ 425g, RT) and the supernatant discarded, and pelleted cells resuspended in 500µl RPMI – 1640 Medium. Cells were then transferred into a 96-well U-bottom plate for staining for flow cytometry analysis.

After resuspension the cells isolated from the paw digestion were plated, 200µl per well, and pelleted by centrifugation (30 secs @ 800g). The supernatant was discarded, cells were resuspended in Zombie (Aqua) in (100µl per well) $DPBS^{-/-}$ then covered and incubated (20 mins, on ice). Following incubation, cells were pelleted by centrifugation and the supernatant discarded. To perform a wash, FACS buffer (100µl per well) was added and cells were resuspended then spun (30 secs @ 800g), this step was then repeated to wash once more. Fc block diluted 1:5 in FACS buffer (50µl per well) was added, cells were resuspended, covered and incubated (15 mins, on ice). After incubation, cells were washed again, pelleted by centrifugation and the supernatant discarded.

For identifying immune cells a panel of (CD11b, CD45, CD64, F4/80, Ly6C, Ly6G, MHC-II and 7/4) fluorescently conjugated antibodies at working dilution in FACS buffer (see table 3) was added (100µl per well), cells were resuspended, covered and

incubated (30 mins, on ice). Cells were washed twice with FACS buffer, pelleted by centrifugation, and the supernatant discarded before finally being resuspended in FACS buffer (200µl) and transferred into FACS tubes.

The panel of antibodies used for this staining includes some that are conjugated to fluorochromes that are tandem dyes. For this reason, extra precautions were taken when staining to ensure that the stability of the tandem is preserved. These dyes are considered less stable than single molecule fluorochromes due to their potential for degradation or decoupling which results in loss of emission from the acceptor and increased emission from the donor. Some tandem dyes are susceptible to cell-mediated damage due to cell metabolism; hence staining was performed on ice. An additional risk to these fluorochromes is fixation. It is recommended that cells (if they must be fixed) are fixed for only for a short period of time due to the risk of over fixing. Therefore, the addition of 4% PFA (50µl), that would normally be used to fix stained cells, was omitted from the protocol when staining with this panel. Consequently, samples were run on an analyser (LSR Fortessa) immediately after staining.

The fluorescently conjugated antibodies specific to the different biomarkers present on the surface of the cell were used to determine subsets of leukocytes (CD45+). Specifically, populations of neutrophils (CD64-Ly6G+) and macrophages (Ly6G-F4/80+) were reviewed. The gating strategy used for the analysis firstly gated on single cell moieties, followed by live cells by exclusion of Zombie positive cells. Hematopoietic cells were then selected as the CD45+ population, which was then used as the parent population for gating on the CD11b+ myeloid cells. The myeloid cell population was then used for gating on specific leukocyte subset populations

#### *2.2.4.4. Histological Staining and Analysis*

Histological staining and analysis were performed for both the 4 day and 40 day experimental arthritis for WT and Gal-1 KO mice. A hind limb joint was removed from each mouse and processed to enable histological analysis (see section 2.4.2.). Briefly, joints were fixed in neutral buffered formalin (NBF) and decalcified in 10% EDTA in $DPBS^{-/-}$ before being trimmed down, embedded in paraffin blocks, sectioned and transferred to slides.

Haematoxylin and Eosin (H&E) staining was performed on deparaffinized sections to assess cell and tissue morphology and distribution including cellular infiltrate and pannus formation. Stained sections were viewed using standard light microscopy (EVOS™ XL Core Imaging System) and images were taken using the 4X objective. The resulting images from the H&E stain were analysed to assess the joints for severity of arthritis, scoring on a scale of zero to three by assessing the bones, cartilage, synovium, level of infiltrate (pannus formation) and overall joint architecture (including thickness of synovium). The analysis was performed blindly against a predetermined scoring system, with a scale from 0-3. A score of zero was given to normal non-arthritic joints displaying normal architecture, with no signs of infiltration or damage. A score of one indicated the joint to be mildly arthritic where synovitis could be observed in the absence of damage. Joints that maintained their architecture but still showed moderate levels of arthritis including synovitis and damage were given a score of two. A top score of three was determined as severely arthritic where there was loss of joint integrity in addition to synovitis and damage.

#### *2.2.4.5. Blood Collection*

Cardiac punctures, a technique suitable for collecting a large and good quality sample of blood from mice, were performed four days post initiation of disease. Isoflurane was used to deeply anaesthetise mice and blood samples were taken from the heart which was accessed via the left side of the chest or from underneath by going through the diaphragm. Blood was withdrawn slowly, to prevent the heart collapsing, using a 25-gauge needle and collected into 1ml syringes. Here syringes were prefilled with 100µl of 100U/ml heparin diluted in saline (dd$H_2O$ + 0.9% NaCl) as an anti-coagulant. In all cases this method was performed as a terminal procedure.

#### *2.2.4.6. Inflammatory Mediator Analysis*

Blood samples (0.5-1ml) were spun (10 mins @ 6000g, RT) and plasma was collected and stored at -80°C. The plasma samples from cardiac punctures were sent to Labospace (LaboSpace Ltd. Milan, Italy) and levels of analytes (see table 7) were measured using a custom designed mouse Luminex screening assay.

## Table 7. Inflammatory mediators analysed in plasma from blood.

| Cytokine | Class | Production | Target Cell/Interaction | Functions | Detection Range (pg/ml) |
|---|---|---|---|---|---|
| IL-1β | Cytokine (pro-inflammatory) | Tissue macrophages, monocytes, fibroblasts, and dendritic cells. Low levels by B lymphocytes, NK cells and epithelial cells. | Macrophages and endothelial cells. | Increases the expression of adhesion factors on endothelial cells to enable transmigration, promoting the recruitment and retention of macrophages. | 75 - 53640 |
| IL-6 | Cytokine (pro-inflammatory) | Secreted by T cells, macrophages, and monocytes to stimulate immune response. | Various cells during acute phase of response. | Regulation of inflammatory chemokines and apoptotic events dictating the profile of leukocyte recruitment. | 10 - 5980 |
| KC (CXCL1) | Chemokine | Induced by inflammatory cytokines (IL-1, TNF, PGE2 and thrombin). | Neutrophils and endothelial cells. | Recruitment of neutrophils to inflammatory sites. | 10 - 7050 |
| MCP-1 (CCL2) | Chemokine | Primarily by monocytes, macrophages, and dendritic cells. | Monocytes and endothelial cells. | Monocyte recruitment into sites of immune responses and cancer. | 60 - 54130 |
| TNFα | Cytokine (pro-inflammatory) | Primarily by monocytes and macrophages. | Macrophages. | Cell adhesion molecule (CAM) and cytokine expression. Induces inflammation and apoptosis. | 0 - 905 |
| $LTB_4$ | Eicosanoid | Leukocytes (in response to inflammatory mediators). | Leukocytes and endothelial cells. | Induce the activation and adhesion of leukocytes on the endothelium, allowing them to bind. | 25 - 2570 |

### *2.2.5. Efferocytosis*

#### *2.2.5.1. Model*

Efferocytosis was assessed by injecting fluorescently labelled apoptotic neutrophils ($2.5 \times 10^6$ cells) into the peritoneal cavity of mice, allowing time for the macrophage to ingest the apoptotic cells and then collecting the cells from the peritoneal cavity for analysis. Efferocytosis was performed with Gal-1 KO mice and WT counterparts, both on a C57BL/6 background.

This model was performed a number of times, the age of mice varied from 8-15 weeks across a number of experiments however in all cases the two genotypes were both age and sex matched for each individual experiment that was performed. Mice were injected with CFSE labelled apoptotic human neutrophils and efferocytosis was assessed following two time points (either 30 or 60 minutes) post injection.

#### *2.2.5.2. Preparation and Injection of Neutrophils*

Human neutrophils were isolated from the peripheral blood of healthy donors by double density centrifugation (as detailed in section 2.3.1.1). Isolated neutrophils were counted and resuspended (to $4 \times 10^6$ cells/ml) in RPMI – 1640 Medium for staining with CFSE. Resuspended cells were added to 50ml falcon tubes (10ml per tube) and CFSE (10µl dissolved in anhydrous DMSO at 5mM) was added at a final concentration of 5µM. Falcon tubes were wrapped in aluminium foil (to protect from light exposure) and incubated (20 mins on a shaker, RT).

Following incubation fluorescence was quenched by addition of an equal volume (10ml) of FBS and the solution mixed by gently inverting the falcon tube several times. The cell solution was spun (10 mins @ 425g, RT), the supernatant discarded, and pelleted cells resuspended in 1ml RPMI – 1640 Medium. A washing step was then performed by topping up the cell solution to 10ml with RPMI – 1640 Medium, pelleting the cells by centrifugation (10 mins @ 425g, RT) and discarding the supernatant. CFSE labelled neutrophils were then counted, resuspended (to 4 x $10^6$ cells/ml) in RPMI – 1640 Medium + 0.5% BSA and transferred in to a T25 culture flask. The flask was then placed upright (to minimise adherence to the surface) and incubated in a humidified chamber in 5% $CO_2$ at 37°C for 20 hours to allow for neutrophils to undergo apoptosis.

After incubation CFSE labelled neutrophils were collected from the culture flask and the cell solution was spun (10 mins @ 425g, RT), the supernatant discarded, and pelleted cells resuspended in 1ml $DPBS^{-/-}$ and counted. Apoptotic CFSE labelled neutrophils were resuspended (to 5 x $10^6$ cells/ml) in $DPBS^{-/-}$ and the cell solution was kept covered.

Administration to mice followed shortly after, 2.5 x $10^6$ cells in 500µl $DPBS^{-/-}$ was delivered into the peritoneal cavity of mice by i.p. injection using a 0.5ml insulin syringe with 27-gauge needle. After 30 or 60 minutes, mice were sacrificed, and peritoneal lavage performed as described in section 2.2.3.1.

#### *2.2.5.3. Analysis by Flow Cytometry*

The peritoneal exudate retrieved was analysed by flow cytometry for efferocytosis. Prior to staining cells with surface antibodies, the cells from the peritoneal exudate were pelleted by centrifugation (5 mins @ 800g, RT), resuspended in 1ml FACS buffer and 200µl was plated into a 96-well U-bottom plate. Steps to pellet the cells, incubate in Fc block, wash cells, stain with antibodies and fix were then performed (as described in section 2.2.3.3).

The fluorescently conjugated antibodies used for the staining included a marker for murine hematopoietic cells (CD45), a murine macrophage marker (F4/80) and a human neutrophil marker (CD66b). Antibodies were added at working dilution (see table 1) in (50µl) FACS buffer. Samples were run on a flow cytometry cell analyser (LSR Fortessa).

To assess levels of efferocytosis the following gating strategy was applied, firstly debris was gated out, followed by selection of mouse haematopoietic cells using the CD45+ population. From this population cells expressing the human neutrophil marker CD66b, which is constitutively expressed on neutrophils independent of cell location, activation or disease state (Lakschevitz *et al.*, 2016), were gated out by selecting the CD66b- population, to exclude macrophages with neutrophils attached to their surface. Murine macrophages were then gated on by selecting the F4/80+ population and the median fluorescence intensity (MFI) of CFSE was then assessed for this population.

## *2.3. In vitro Methods*

### *2.3.1. Human Blood Leukocytes*

Human leukocytes were isolated from the peripheral blood of healthy volunteers using double density centrifugation. All donors gave informed consent and all work was performed under ethical approval (QMREC2014.61) granted by the QMUL Research Ethics Committee.

Collected blood was handled quickly to prevent coagulation and steadily to avoid activation of the cells, likewise solutions that mix with the blood were warmed to 37°C prior to use.

#### *2.3.1.1. Neutrophil Isolation*

Blood was drawn from the median cubital vein using a 21-gauge butterfly needle and collected into a 60ml syringe. Blood was gently expelled from the syringe into a sterile pot containing an anti-coagulant. Specifically, 3.2% (w/v) sodium citrate (dissolved in $dH_2O$) was used at a 1:10 ratio of anti-coagulant to blood volume. The pot was slowly tilted several times to disperse the anti-coagulant throughout the blood sample and RPMI – 1640 Medium was added (in equal volume to blood).

The diluted blood (6ml per 15ml falcon tube) was then gently layered above a double density Histopaque layer. To create the double density Histopaque layer two solutions (composing of polysucrose and sodium diatrizoate) adjusted to specific densities (1.077 g/ml and 1.119 g/ml) were layered one above the other. Firstly Histopaque –

1119 (3ml) was added to the bottom of (15ml) falcon tubes, above which Histopaque – 1077 (3ml) was carefully added to create two separate layers. The blood and Histopaque was then spun (30 mins @ 400g, RT). Following the spin, separate layers of leukocytes, plasma and Histopaque were visibly formed (figure 12).

Following centrifugation (30 mins @ 400g, RT) the neutrophil layer was collected from each of the tubes, transferred to new (15ml) falcon tubes and RPMI – 1640 Medium was added (in equal volume - 6ml of each). The blood and media were then spun (15 mins @ 425g, RT) and the supernatant discarded. The remaining red blood cells were then lysed using ice cold water (7.5ml) and falcon tubes were gently inverted (10 seconds). To return solution to an isotonic balance 3.6% (w/v) sodium chloride (dissolved in $dH_2O$) was added at a 1:3 ratio of salt solution to cell solution. The solution was then spun (10 mins @ 425g, RT), the supernatant was discarded and remaining pelleted neutrophils were resuspended in RPMI – 1640 Medium (1ml) and counted using Turk's Solution. The neutrophil suspension was added to Turk's Solution (10µl to 990µl respectively) to give a final dilution of 1:100 and cells were counted on a Neubauer counting chamber (as described in section 2.2.3.3). Following the count neutrophils were resuspended in media (type and volume dependant on protocol) to the required number of cells per ml.

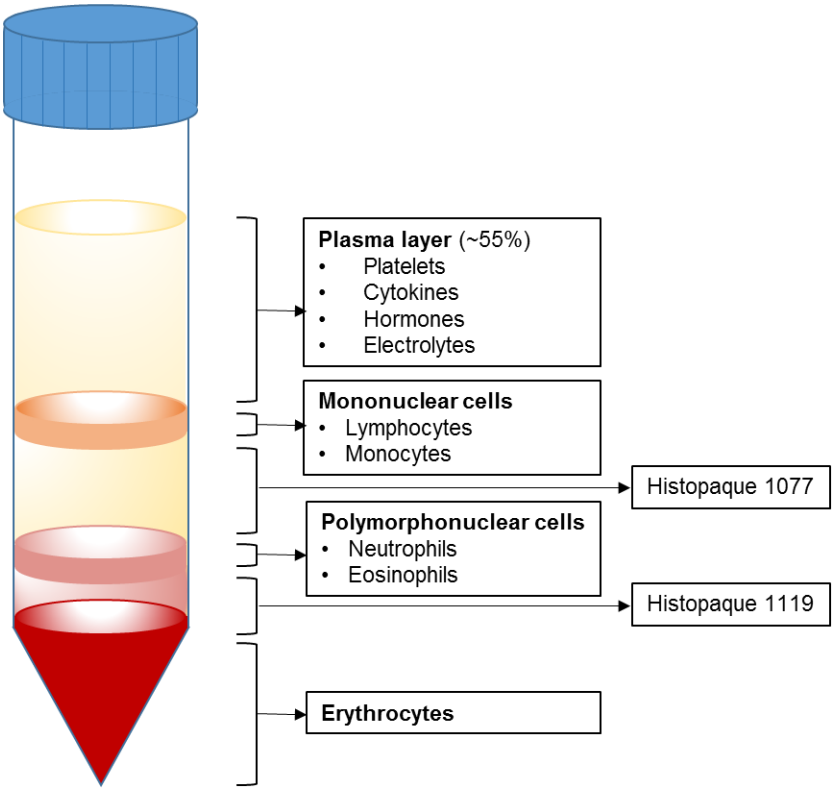



**Figure 12. Leukocyte separation using double density centrifugation.**
Whole blood was layered above two different density histopaque solutions (1.077g/ml and 1.119g/ml). Following centrifugation leukocyte subsets separate out according to their density and the density of the solutions. Plasma cells, mononuclear cells, polymorphonuclear cells and erythrocytes form individual layers, as shown, due to their respective increase in cellular density. The separate layers are then collected dependent on cell type of interest.

#### *2.3.1.2. Monocyte Isolation*

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy cones. Cones were obtained in collaboration with a hospital-based phlebotomy facility and were produced from blood drawn from healthy donors. The cone, also known as a Leukocyte reduction system chamber (LRSC) is the fraction of blood leftover following platelet collection. The platelet rich fraction obtained from centrifugation of whole blood (450ml) will also contain leukocytes, which are then filtered out by leukoreduction and collected into an LRSC. The LRSC will contain a high density of leukocytes with a low concentration of neutrophils (relative to both the PBMC population and proportion present in peripheral blood).

Each buffy cone contained 20ml of blood which was collected into a 50ml falcon tube. The cone was pointed upwards and the tubing coming from the tip was cut, the open end was put into the falcon and the cone inverted so that blood drained into the falcon. The tubing attached to the other broader end was cut and a 21-gauge needle inserted. A 30ml syringe containing 20ml $DPBS^{-/-}$ was attached to the needle and flushed through the cone. The syringe was then refilled with air and the cone was flushed to release any remaining liquid. The blood (diluted 1:1 with $DPBS^{-/-}$) was then gently layered above 10ml Histopaque – 1077 in new 50ml falcon tube. The blood and Histopaque was then spun (30 mins @ 400g, RT) to separate out layers of leukocyte subsets from plasma.

Following centrifugation, the PBMC layer was collected from the tube, transferred to a new (50ml) falcon tube and $DPBS^{-/-}$ (to 50ml) was added. The diluted PBMCs were then spun (10 mins @ 425g, RT) and the supernatant discarded.

*2.3.1.3. Monocyte Derived Macrophages*

PBMC were isolated from a buffy cone (as in section 2.3.1.2.), following the final centrifugation the pelleted PBMC were resuspended in $DPBS^{+/+}$ (15ml) and counted using Turk's Solution (as described in section 2.2.3.3). PBMC were then resuspended to 30 x $10^6$/ml in $DPBS^{+/+}$ and plated into 10cm bacteriological dishes, 1ml cells added to 6ml $DPBS^{+/+}$ to give a final concentration of 30 x $10^6$ cells in 7ml $DPBS^{+/+}$ per plate.

The plate was then incubated in a humidified chamber with 5% $CO_2$ at 37°C for one hour to allow monocytes to adhere to the plate. Adherence was confirmed using standard light microscopy and the solution aspirated. Cells were then washed three times with $DPBS^{-/-}$ (5ml per plate added, swirled around the plate, and aspirated) and then recovered with RPMI – 1640 Medium + 20% FBS + 50ng/ml hrM-CSF + 1% P/S. The plate was then incubated in a humidified chamber in 5% CO2 at 37°C for six days (with media refreshed on day 3) to allow for monocytes to differentiate to macrophages.

To detach macrophages from the plate, culture medium was aspirated, and plates were washed with $DPBS^{-/-}$ (as described above). Warmed (37°C) accutase + 0.5mM EDTA (3ml) was overlaid and cells were incubated in a humidified chamber in 5% $CO_2$ at 37°C for 20 minutes to allow macrophages to detach. After the incubation period a cell scraper was gently swept across the bottom of the plate to detach the cells and the plate flushed several times with the accutase solution using a 3ml pasteur pipette. The cells in solution were then collected, added to a (15ml) falcon tube and the plate checked under a light microscope to confirm detachment of macrophage from the bottom of the plate. $DPBS^{-/-}$ (3ml) was then added to the plate and used to flush around

the outsides several times to collect any remaining cells. Falcon tubes were topped up to 15ml with $DPBS^{-/-}$, spun (5 mins @ 425g, RT) and the supernatant discarded leaving the pelleted macrophages.

### *2.3.2. Neutrophil Apoptosis*

Neutrophils isolated from human peripheral blood (as in section 2.3.1.1.) were treated with hrGal-1 (0.03, 0.1, 0.3 and 1μM) ± human recombinant Granulocyte Macrophage Colony Stimulating Factor (GM-CSF, 50ng/ml), which is known to delay neutrophil apoptosis (Colotta *et al.*, 1992). In additional assays, instead of GM-CSF, neutrophils were treated with serum amyloid A (SAA, 10μg/ml), an apolipoprotein in plasma likewise reported to delay the spontaneous apoptosis of neutrophils (Christenson *et al.*, 2008).

Neutrophils were incubated for 20 hours and levels of apoptosis were primarily determined by AnnexinV (AnxV) binding and Propidium Iodide (PI) staining using flow cytometry. Samples were also stained with 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$), a lipophilic dye that is selective for the mitochondria of live cells and cytospins of viable (0h) and apoptotic (20h) cells performed, to provide accompanying methods for the quantification of apoptosis.

To determine whether Gal-1 influences initial PS exposure neutrophils isolated from human peripheral blood were also assessed by AnxV/PI staining at 1h and 4h (± GM-CSF (50ng/ml)) timepoints.

Neutrophils were resuspended (2 x $10^6$ cells/ml) in RPMI – 1640 Medium + L-glutamine (L-glut, 2mM) + 5% autologous serum. Autologous serum was collected from neutrophil donors by transferring blood into 1.1ml Z-Gel Micro tubes containing a gel matrix specialised for serum separation. Tubes were spun (10 mins @ 10000g, RT) and as a result the blood cells pelleted below the gel matrix and the serum remained above. The serum was collected from the tubes and placed on ice until required for media preparation.

Neutrophils were then transferred in to a 96-well u-bottom plate (200µl per well) and treated as described above. The plates were then incubated in a humidified chamber in 5% $CO_2$ at 37°C for 20 hours to allow for neutrophils to undergo apoptosis.

#### *2.3.2.1. AnxV/PI*

After incubation cells were pelleted by centrifugation (30 secs @ 800g), the supernatant was discarded, and pelleted cells stained for AnxV/PI. AnxV binds to PS on the neutrophil extracellular membrane, whereas PI is a membrane permeable dye able to enter the cell when there is a loss of outer membrane integrity.

AnnexinV/PI in AnnexinV binding buffer (50µl) was added to each well. Cells were resuspended, covered and incubated (15 mins, RT). AnnexinV binding buffer (150µl) was then added to each well and the contents transferred to FACS tubes. Cells were analysed by flow cytometry immediately after staining. Apoptosis of neutrophils was determined by analysing the AnnexinV/PI staining of single cells by quadrant gating on the viable, early apoptotic, late apoptotic and necrotic populations (figure 13).

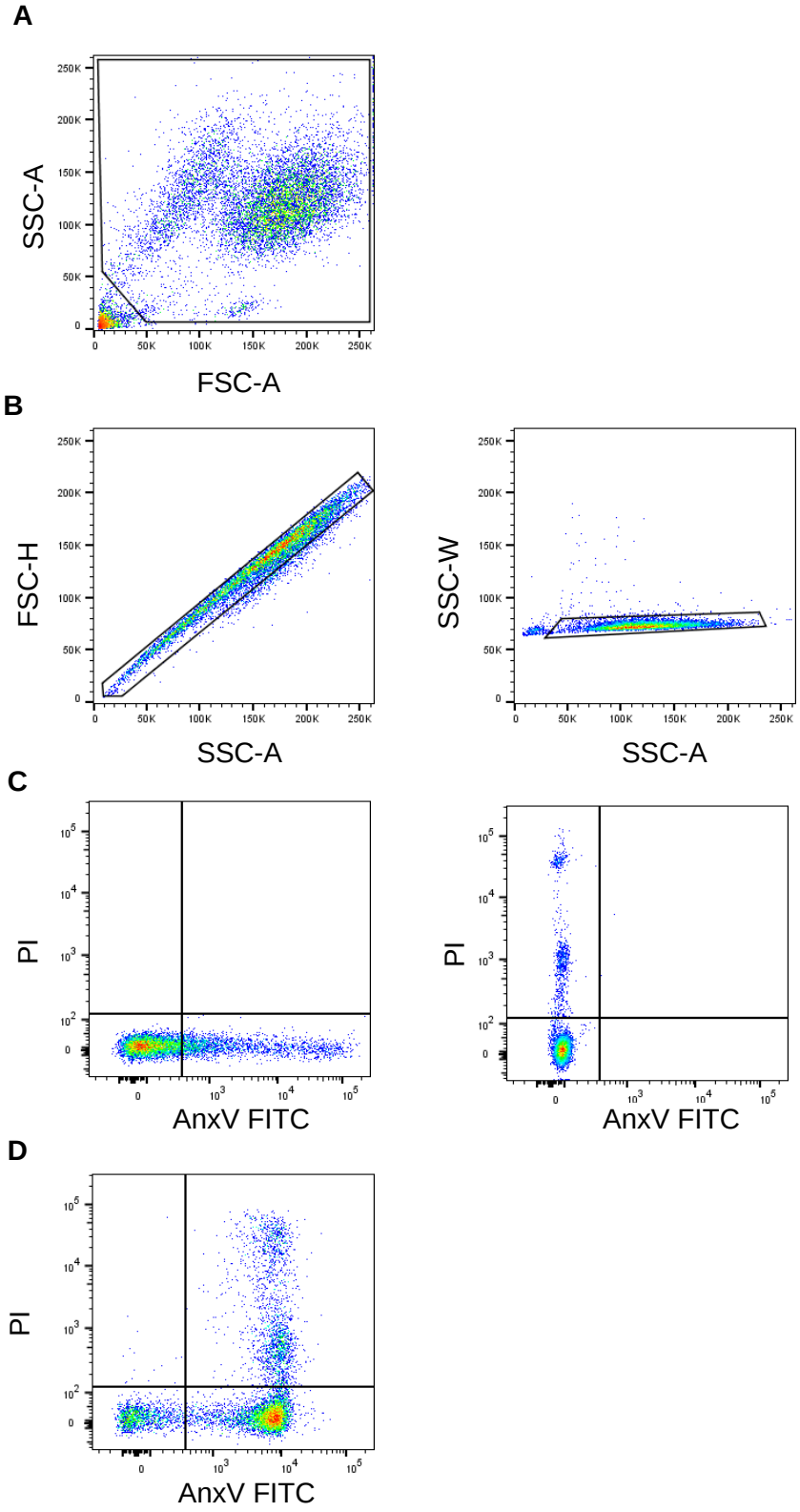



**Figure 13. *In vitro* neutrophil apoptosis gating strategy.**
(A) Human neutrophils isolated from peripheral blood were displayed on a Forward Scatter Area (FSC-A) against Side Scatter Area (SSC-A) plot and gated on to exclude debris. (B) Single cell moieties were obtained from the population by first plotting FSC-A against Forward Scatter Height (FSC-H) to exclude doublet cells, followed by further doublet exclusion by plotting this population on a SSC-A against Side Scatter Width (SSC-W) axis. (C) Neutrophils that were single stained for either AnnexinV (AnxV) or Propidium Iodide (PI) were used to apply gating. (D) The population of singlets was plotted on an axis with the bandpass filter for AnxV against PI. The subsequent percentages of neutrophil cell state were calculated using this quadrant gating strategy.

### *2.3.2.2. Nuclear Morphology*

Cytospins were prepared by centrifuging (30 secs @ 800g) the incubated plate and resuspending the pelleted cells in $DPBS^{-/-}$ (100µl). Samples (50µl) were then added to a cytospin chamber and cells were spun for 3 mins @ 200g. Slides were removed and left to air dry prior to staining.

Once dry cells were fixed with methanol, left to air dry and then stained using Kwik-Diff Staining System. Specifically, neutrophils were exposed to solution 2 for three minutes, after which it was then tipped off the slide. Solution 3 was then pipetted on and left for five seconds, followed by a wash, whereby distilled water was pipetted on and immediately tipped off several times until running off the slide clear. Slides were left to air dry and a coverslip applied using Entellan mounting medium. Once dry, stained slides were visualised under light microscopy using the 60x objective.

To provide differential cell counts 200 cells were counted on each cytospin and apoptotic neutrophils, determined morphologically by the condensed nuclei as shown in figure 14, were counted and expressed as a percentage of the total neutrophil count.

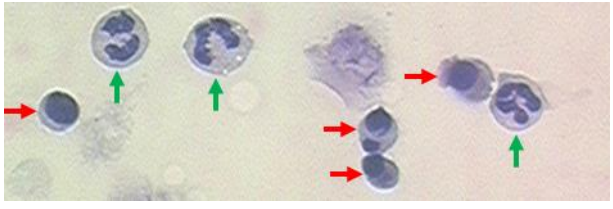


**Figure 14. Neutrophil nuclear morphology.**
Cytospins of human neutrophils isolated from peripheral blood were stained using Kwik-Diff Staining System and visualised under light microscopy using the 60x objective. Nuclear morphology was assessed to determine cell viability. Neutrophils were considered viable, indicated by the green arrows, when a multi-lobular nucleus was present whereas apoptotic neutrophils, indicated by the red arrows, were determined by their visibly condensed nuclei.

#### *2.3.2.3. $DiOC_6$*

After incubation cells were pelleted by centrifugation (30 secs @ 800g), the supernatant was discarded and pelleted cells stained for $DiOC_6$, a lipophilic dye that stains the organelle membranes within live cells. The cell permeant dye has particularly high specificity for the mitochondrial membrane and thus can be used to determine the depletion of mitochondrial membrane potential that occurs as cells undergo apoptosis.

For $DiOC_6$ staining a working concentration of 40nM of dye was added to cells. This was prepared from a 4µM stock solution of $DiOC_6$ (dissolved in EtOH) that was further diluted (1:100) in RPMI – 1640 Medium. Pelleted cells were then resuspended in the diluted dye (200µl per well), covered and incubated in a humidified chamber in 5% $CO_2$ at 37°C for 30 minutes. After this the plate was spun (30 secs @ 800g), supernatant was discarded and pelleted cells were washed in FACS buffer (100µl), the washing steps were then repeated. Finally, the cells were resuspended in FACS buffer (200µl), transferred to FACS tubes and analysed immediately by flow cytometry. Apoptosis of neutrophils was determined by analysing the $DiOC_6$ staining of single cells, by gating on the viable ($DiOC_6$ high) population of cells.

#### *2.3.2.4. Signalling Pathways*

Intracellular signalling pathways known to be involved in neutrophil apoptosis were investigated by Western blot (see section 2.4.4.). Neutrophils ± hrGal-1 (1µM) ± GM-CSF (50ng/ml) were incubated at 37˚C (in a water bath) for 1, 5 and 15 mins prior to

being immunoblotted for the phosphorylated forms of AKT, ERK and p38 MAPK as well as their respective totals.

For this assay neutrophils were isolated from human peripheral blood (as in section 2.3.1.1.) with the omission of the red cell lysis step to reduce neutrophil activation and resuspended to 2 x $10^6$ neutrophils in 150µl RPMI – 1640. Following stimulations neutrophils were lysed with 50µl hot Western blot loading buffer (LDS + DTT, 95˚C) and a standard Western blot protocol followed (see section 2.4.4.).

### *2.3.3. Neutrophil AnnexinA1 Expression*

Annexin A1 (AnxA1) is an endogenous protein that exerts anti-inflammatory and pro-resolving effects over many cellular events, thus limiting the extent of the inflammatory response. Contained within neutrophil gelatinase granules, endogenous AnxA1 is rapidly translocated to the cell surface upon activation (Perretti and D'Acquisto, 2009), furthermore it can override pro-survival signals to induce neutrophil apoptosis (El Kebir *et al.*, 2008).

This assay set out to determine if incubation of neutrophils ± GM-CSF ± hrGal-1 modulates the levels of cellular AnxA1 during apoptosis. Neutrophils isolated from human peripheral blood (as in section 2.3.1.1.) were diluted in culture media (2 x $10^6$ cells/ml in RPMI 1640 + L-Glut + 5% autologous serum) treated with hrGal-1 (1µM) ± GM-CSF (50ng/ml) in a 96-well u-bottom plate (200µl per well) and incubated at 37°C 5% $CO_2$ for 4h or 20h. After incubation cells were pelleted by centrifugation (30 secs @ 800g) and the culture media was collected. Neutrophils were then resuspended in

FACS buffer and samples divided equally between two 96-well u-bottom plates, to assess surface and total staining.

#### *2.3.3.1. Surface Expression*

Neutrophils were stained for surface AnxA1 using an unconjugated mouse anti-human AnxA1 primary antibody diluted with blocking immunoglobulins (1:1 in FACS buffer, 50µl per well) and incubated (30mins, on ice). Neutrophils were then pelleted by centrifugation (30 secs at 800g) and the supernatant discarded. Cells were washed twice in FACS buffer (200µl) and a fluorescently conjugated anti-Mouse secondary antibody was added, and cells incubated (30 mins, on ice). Finally, cells were washed twice in FACS buffer and stained neutrophils were collected (in 200µl FACS buffer per well), transferred to FACS tubes and fixed with (50µl) 4% PFA. Samples were acquired (on an LSR Fortessa), the single cell populations were selected and their MFI of the conjugated secondary to the AnxA1 primary antibody was assessed.

#### *2.3.3.2. Total Expression*

Total neutrophil AnxA1 was assessed using a standard protocol for fixation followed by permeabilisation of cells. Pelleted neutrophils were resuspended in (200µl) Fixation buffer and incubated (30 mins, at RT). Cells were then pelleted by centrifugation and the supernatant discarded followed by two wash cycles with permeabilisation buffer (200µl). An unconjugated mouse anti-human AnxA1 primary antibody was added in permeabilisation buffer (50µl per well) and cells were incubated (30 mins, at RT). After incubation two wash cycles with permeabilisation buffer (200µl) were performed. An

anti-Mouse secondary antibody conjugated with a fluorochrome was added (50µl per well) in permeabilisation buffer and cells were incubated (30 mins, RT). Incubation was followed by three wash cycles with permeabilisation buffer (200µl) after which cells were resuspended in FACS buffer (200µl) and transferred into FACS tubes. Samples were acquired and analysed as above (in section 2.3.4.1).

#### *2.3.3.3. Release*

To detect release of AnxA1 from neutrophils, cell free culture media was collected after 4h and 20h incubation and analysed by Western blot (see section 2.4.4.). Following incubation cells were pelleted by centrifugation and the cell culture media was collected in to Eppendorfs. A subsequent centrifugation (5min @ 400g) was performed to ensure media was free from cells or cellular debris. Loading buffer (LDS + DTT, see section 2.4.4.1.) was diluted 1:4 with the collected supernatant (5µl buffer to 15µl media) and samples were incubated at 95˚C on a heat block for 5 minutes. Prepared samples were then stored at -20˚C prior to loading 10µl and following a standard Western blot protocol (see section 2.4.4.2. onwards).

### *2.3.4. Neutrophil Galectin-1 Expression & Binding*

Extracellular Gal-1, exogenously bound and/or endogenously expressed, was detected on neutrophils using flow cytometry. This assay was performed to determine the relative levels of Gal-1 on the surface of neutrophil as they move through the process of apoptosis. Likewise it was also used to establish if the Gal-1 detected on the surface was coming from the addition of the exogenous protein binding to the cell or if it originated intracellularly and if its exposure at the cell surface was modulated as

a consequence of undergoing apoptosis. Whether the exogenous Gal-1 had a higher affinity for binding the viable or apoptotic neutrophils was also addressed as was whether the bound Gal-1 would again dissociate from the cell surface in the presence of a competitive binding sugar.

Neutrophils isolated from human peripheral blood (as in section 2.3.1.1.) were diluted in culture media (2 x $10^6$ cells/ml in RPMI 1640 + L-Glut + 5% autologous serum) treated ± hrGal-1 (1µM) ± GM-CSF (50ng/ml) in a 96-well u-bottom plate (200µl per well) and incubated at 37°C, 5% $CO_2$. Surface Gal-1 was assessed after 1h, 4h and 20h incubation. Serum free incubations and addition of lactose (30mM) were also included as further controls in some assays.

Following the incubation period neutrophils were pelleted by centrifugation (30 secs @ 800g), the supernatant was discarded, and cells surface stained for Gal-1 using an unconjugated primary antibody to human Gal-1 followed by a conjugated secondary to the antibody host. The anti-hGal-1 primary was diluted to working concentration (table 1) in FACS buffer with blocking immunoglobulins (1:1), 50µl was added to each well and neutrophils were resuspended and incubated (30mins, on ice). Neutrophils were then spun, and the supernatant discarded followed by two washes with (200µl) FACS buffer. Cells were then resuspended in FACS buffer (50µl per well) containing fluorescently conjugated anti-Goat secondary antibody and incubated (30 mins, on ice). Cells were then washed twice in FACS buffer before being resuspended in (200µl) FACS buffer, transferred to FACS tubes and fixed with (50µl) 4% PFA. Following sample acquisition, data was analysed by selecting the single cell

populations and measuring the MFI of the conjugated secondary to the hGal-1 primary antibody.

### *2.3.5. Neutrophil Galectin Receptor Expression & Binding*

Galectins bind specific transmembrane glycoproteins expressed on nucleated haematopoietic cells. Assessment of receptor expression on neutrophils was performed ± GM-CSF to investigate whether the galectin binding glycoproteins on the cell surface, were modified in response to this pro-survival cytokine. In this set up neutrophils were also incubated ± hrGal-1 to determine if there was any alteration to the binding capacity of Gal-1.

Neutrophils isolated from human peripheral blood (as in section 2.3.1.1.) were diluted in culture media (1 x $10^6$ cells/ml in RPMI 1640 + L-Glut + 5% autologous serum) treated with hrGal-1 (0.03µM, 0.1µM, 0.3µM and 1µM) ± GM-CSF (50ng/ml) in a 96-well u-bottom plate (200µl per well) and incubated at 37°C for 4 hours. Neutrophils were then pelleted by centrifugation (30 secs @ 800g), the supernatant discarded and then surface stained for CD43, CD44 and CD45, three known Galectin receptors (reviewed in (Rabinovich *et al.*, 2002)).

Cells were resuspended in 40µl per well mix of fluorescently conjugated antibodies to CD43 and CD45 and an unconjugated primary antibody for CD44. Antibodies were diluted to working dilution (table 3) in FACS buffer with blocking immunoglobulins (1:1) and incubated (1h, on ice). Neutrophils were then washed three times in FACS buffer followed by incubation (30 mins, on ice) with 40µl per well fluorescently conjugated anti-rat secondary. Cells were then washed three times, resuspended in 200µl FACS

buffer and fixed with 50µl 4% PFA. Galectin receptor levels were determined by selecting the single cell populations and analysing the fluorescence intensity of each.

### *2.3.6. Efferocytosis*

#### *2.3.6.1. Model*

Efferocytosis was assessed by overlaying human neutrophils (2 x $10^6$ or 5 x $10^6$ cells in 1ml) on human macrophages (1 x $10^6$ cells/well), allowing time (1h) for the macrophage to phagocytose the cells and then collecting the cells from the well for analysis by flow cytometry.

The protocol was used to assess whether neutrophils undergoing apoptosis ± hrGal-1 (1µM) ± GM-CSF (50ng/ml) would be efferocytosed any differently as a result of either agent. The assay was also performed with two different macrophage:neutrophil ratios for consideration of macrophage efferocytic satiation. Furthermore, the addition of hrGal-1 (1µM) to both viable and apoptotic neutrophils (1h) prior to and during their addition to macrophages was used to determine whether Gal-1 served as an opsonin.

#### *2.3.6.2. Preparation of Macrophage*

Monocytes were isolated from buffy cones (as in section 2.3.1.2.) and differentiated to macrophage (as in section 2.3.1.3). Following centrifugation macrophages were counted and diluted to 1 x $10^6$/ml in media (RPMI – 1640 Medium + 20% FBS + 1% P/S), plated (1ml per well) in a 6-well cell culture plate and incubated overnight at 37°C with 5% $CO_2$.

### *2.3.6.3. Preparation and Incubation of Neutrophils*

Neutrophils were isolated from human peripheral blood (as in section 2.3.1.1.) and assayed in one of the following ways:

- Cultured in media (RPMI 1640 + L-Glut + 5% autologous serum) ± hrGal-1 (1μM) ± GM-CSF (50ng/ml) and incubated (at 37°C 5% $CO_2$) overnight. Apoptotic cells were then collected, counted and resuspended to either 2 x $10^6$ cells/ml or 5 x $10^6$ cells/ml in media (RPMI 1640 +1% FCS).
- Cultured in media (RPMI 1640 + L-Glut + 5% autologous serum) and incubated (at 37°C 5% CO2) overnight. Apoptotic cells were then collected, counted and resuspended to 2 x $10^6$ cells/ml (RPMI 1640 +1% FCS) ± 1μM hrGal-1. Apoptotic cells were incubated (at 37°C, 5% $CO_2$) for a further hour prior to overlay on macrophages.
- Freshly isolated neutrophils resuspended to 2 x $10^6$ cells/ml were incubated (at 37°C, 5% $CO_2$) for 1h in media (RPMI 1640 +1% FCS) ± 1μM hrGal-1 prior to overlay on macrophages.

### *2.3.6.4. Analysis by Flow Cytometry.*

Following the 1h co-incubation of neutrophils and macrophages media was aspirated, and the wells were washed with $DPBS^{-/-}$. Macrophages were collected using accutase + 0.5mM EDTA (1ml) as described previously (see section 2.3.1.3).

Pelleted cells were resuspended in FACS buffer (500μl) and 200μl was plated into a 96-well U-bottomed plate. For staining a standard protocol for fixation followed by permeabilisation of cells was used as described previously (see section 2.3.3.2). Cells

were stained for the human neutrophil and macrophage biomarkers, CD66b and CD86 respectively. Samples were run on a Flow cytometer (LSR Fortessa) and efferocytosis was confirmed as cells double positive for both the macrophage and neutrophil specific biomarkers. The MFI of the anti-CD66b antibody was used to compare the relative quantity of neutrophils engulfed by individual macrophages.

## *2.4. Supporting Protocols*

### *2.4.1. Flow Cytometry*

Flow cytometry is a high throughput laser-based method used for the purposes of counting, sorting and profiling cells (and other particles). It is used extensively and is a common approach for whole blood analysis, isolation and characterisation of rare populations and immunophenotyping, as well as for assessing cell cycle and cell viability.

#### *2.4.1.1. The Flow Cytometer*

The flow cytometer consists of three core systems: fluidics, optics and electronics and has the ability to analyse thousands of particles per second. The fluidics system is responsible for the hydrodynamic focusing and includes the flow cell, the site where the sample and sheath fluid are injected and combined to ensure the entities in the sample are carried in single file. The sample then reaches the laser intercept which precedes the optic system within the machine. Multiple sources, filters and detectors of light make up the optic system, where laser beams of various wavelengths are used to screen the sample, if these provoke ‘excitation’ of elements within the sample then ‘emission’ of light follows.

#### *2.4.1.2. Acquiring Sample Populations*

Forward light scatter (FSC), side light scatter (SSC) and fluorescence are the key parameters detected and measured by the machine, with each providing distinct information. Particle size can be understood using the FSC parameter as larger

entities will produce more forward scattered light. As opposed to FSC, which is light that continues to travel in the same direction as its original path regardless of making contact, SSC is the light that is refracted in different directions following contact and can be used to discern the complexity of the entity. Specifically, SSC provides information on granularity whereby a positive correlation links the two.

Populations can often be separated based on FSC and SSC alone, however it is often more robust a method to do so using protein expression. The most common approach to this involves staining with fluorescently conjugated antibodies specific to cell surface antigens. A blocking step, with recombinant Fc protein, is used to reduce false positive (non-specific) results and high fluorescent background that can result from immunoglobulin (Ig) binding non-specifically to Fc receptor expressing cells.

#### *2.4.1.3. Compensation*

Fluorescence compensation is required when there is spectral overlap between fluorophores which occurs when, in addition to falling within its selected filter, there is also a ‘spill over’ of fluorescein into other filters/channels. Each individual fluorophore will emit photons of multiple energies and wavelengths, as a result, many of the fluorophores used in flow cytometry will have some degree of overlapping emission spectra and moreover emission from a single fluorophore will be measured by multiple detectors. To address the measurement of the photons of one fluorophore in multiple detectors, the spill over from the primary signal into a secondary channel must be corrected for, this is referred to as compensation.

Compensation is a mathematical method aimed at ensuring that erroneous contributions of fluorochromes don't affect the distribution of the data. Single stained samples are used to reveal the amount of spectral overlap, thus by running a positively stained single sample for each fluorochrome the amount of spill over is revealed. The values obtained can be combined and mathematically converted into a compensation matrix (Bagwell and Adams, 1993).

Most often it is considered best practice to use automatic compensation, provided by either a package on the flow cytometer itself or preferably by third-party software, due to the complexity of the methodology.

#### *2.4.1.4. Compensation Samples*

Compensation beads is often an advantageous approach to obtain the required samples. Although cells can in many cases be used successfully as compensation controls, risks such as low antigen expression or cell number can result in the staining not being bright enough and/or not acquiring enough events.

In the current study, compensation controls have been prepared using compensation beads, specifically UltraComp eBeads™, which compose a mix of positive and negative beads. Within the suspension the positive beads are reactive to hamster, mouse and rat antibodies and provide a positively stained population, the negative beads are non-reactive and will not bind antibody, thus serving as a negative (un-stained) population.

To prepare compensation samples using the beads Eppendorf tubes containing 20$\mu$l of beads and 100$\mu$l FACS buffer are prepared for each of the antibodies, 1$\mu$l of an antibody is added in singular to each of the tubes and the solution gently vortexed. The antibody and bead solution are then incubated in the dark at room temperature for 20 minutes. Following incubation an additional 1ml of FACS buffer was added to the tubes which were then spun (5 mins @ 500g, RT) in a micro-centrifuge. FACS buffer was aspirated and the pelleted beads resuspended in (250µl) fresh FACS buffer. Samples were then stored at 4°C before being ran on a flow cytometry cell analyser (LSR Fortessa).

### *2.4.2. Histology*

#### *2.4.2.1. Fixation with Neutral Buffered Formalin*

A hind leg from each mouse was collected into a solution of 10% NBF (10% formaldehyde (w/v), 0.4% sodium phosphate monobasic (w/v) and 0.65% sodium phosphate dibasic (w/v) dissolved in dd$H_2O$ and equilibrated to pH 7.4), a solution considered to be more effective than other simple formalin mixtures. The phosphate salts present in NBF offer a protective effect for erythrocytes during the fixation process and the formation of formalin pigment is prevented by the neutral pH of the solution.

The fixative volume was approximately 20 times the volume of the tissue being fixed, samples were kept sedentary in solution for 48 hours (at room temperature). Tissues must have sufficient time in fixation solution to allow for the chemical reactions required for crosslinking to occur. Crosslinking is essential to provide the cellular and fibrous elements protection from damage that may be caused using decalcifying agents and dehydrating ethanol in later steps.

#### *2.4.2.2. Decalcification with Ethylenediaminetetraacetic Acid*

Following fixation, the samples were then placed in a solution of 10% EDTA (w/v) pH 7.4. The EDTA acts as a chelating agent, facilitating the formation of two or more coordinate bonds between a multiple bonded ligand and a single atom, after being bound by EDTA, calcium ions will remain in solution, but reactivity is diminished. Routinely refreshing the EDTA is essential for the continual removal of calcium ions,

consequently the solution was changed 1-2 times per week continuously until the tissue samples had thoroughly decalcified. The process was performed at room temperature and was aided by standing the samples in solution on a shaker to provide gentle agitation. Thorough decalcification was determined by physically testing the pliability of the joint. Firstly, feeling the specimen for remaining calcified areas followed by gentle manipulation and secondly by piercing the bone with a fine needle. The bone bending without resistance and the needle penetrating the bone indicated the end point of the process.

#### *2.4.2.3. Paraffin Embedding and Sectioning*

After the decalcification process the leg was sectioned further. Separating the ankle and knee joint segments from each other, the ankle section was then cut through the sagittal central plane. The result of this was a roughly cube shaped section of the knee joint as well as two equal sections containing the ankle joint (figure 15).

The prepared samples were placed in individual cassettes, remaining acid was washed out by overnight storage in dd$H_2O$, following which cassettes were put in to 70% EtOH and sent for paraffin infiltration. This procedure dehydrates the tissue through a series of graded ethanol baths to displace the water, before being infiltrated with wax in a tissue processor (Leica). The tissue samples were then embedded into moulds using hot paraffin wax and the resulting blocks left to cool and harden on a cold plate (-4°C), solidified blocks were then removed from their moulds. These went on to be trimmed and sectioned on the microtome (Leica) to a thickness of 5µm and were transferred to charged glass microscope slides and left to dry.

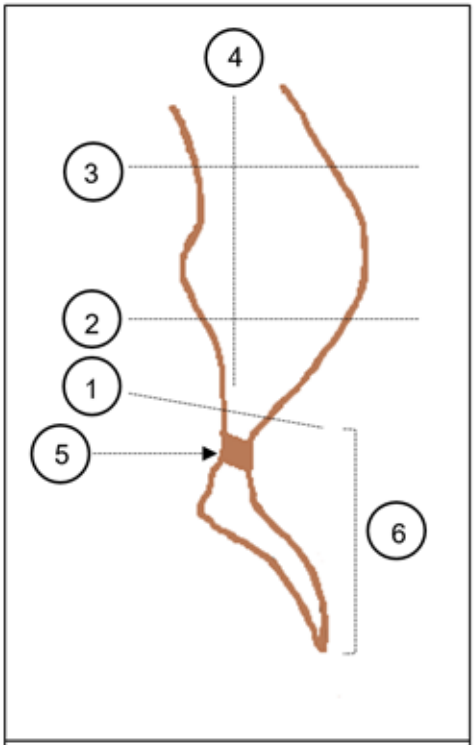



Image indicates location of dissections described (see right).

1. Make a transverse cut with a scalpel
   → Separating paw and knee joint segments.
2. Make a transverse cut below the patella, ensuring synovial joint remains intact and in form.
   → Inferior to joint capsule.
3. Make a transverse cut above the patella, ensuring synovial joint remains intact and in form.
   → Superior to joint capsule.
4. Make a cut to produce a specimen of a roughly cube shape.
   → Down the longitudinal plane.
5. From the paw remove the 'sock' (rim of hair/skin).
6. Make a cut, as close to the midline as feasible, dividing the paw in to equal left and right sides, and taking the incision through the centre of the ligand at the back of the joint also.
   → Along the sagittal plane.

**Figure 15. Procedure for sectioning of decalcified joint.**
The procedural steps (1-6) taken to section the decalcified joint are displayed. Six cuts are made along different planes of the joint. The section that results is a roughly cube shaped knee joint section as well as two equal sections containing the ankle joint.

#### *2.4.2.4. Haematoxylin and Eosin Staining*

Before staining, dried slides were placed on a slide heater at 65°C to soften the wax, samples were then deparaffinised using Histo-Clear®, into which slides were submerged for two incubations of five minutes each. Sections were rehydrated by passing them through alcohols of depreciating concentrations, from anhydrous EtOH to 95%, before being exposed to distilled water, with five-minute incubations in each solution. The specimens being stained for H&E were exposed to Haematoxylin for five minutes, followed by five minutes rinsing in running tap water and then Eosin Y solution for two minutes. After the dye samples were again rinsed for five minutes in running tap water before being dehydrated using a series of increasing concentrations of alcohols. Slides were exposed to solutions of 70%, 90% and 100% EtOH and spent a period of five minutes in each solution. Finally, samples were cleared by two submersions in Histo-Clear® for 5 mins each, left to air dry and a coverslip applied using the xylene based mounting medium Entellan.

Stained H&E slides exhibit blue nuclei and pink cytoplasm, this results from the ionic bonding between ions of opposite charges, one fixed in the tissue and the other in the dye. Haematoxylin is positively charged (cationic) and will bind to negatively charged elements including nucleic acids in the nucleus. Eosin is negatively charged (anionic) and will react with positively charged tissue components, including amino groups in cytoplasm proteins.

### *2.4.3. Genotyping*

The genotype of mice from the Gal-1 KO colony was confirmed using a standard polymerase chain reaction (PCR) method. A technique that amplifies the required DNA sequence generating many copies from a small amount of the original DNA segment/gene. The amplified DNA was then separated by electrophoresis alongside a molecular weight marker, which can subsequently be used to determine the base pair (bp) number. Due to the use of sequence specific primers the selected strands of DNA are different lengths for WT and Gal-1 KO mice, therefore presence or absence of DNA fragments of specific bp length can be used to verify the genotype of the mice.

The REDExtract-N-Amp Tissue PCR Kit (containing Tissue Preparation Solution, Extraction Solution, Neutralising solution and REDExtract-N-Amp PCR Reaction Mix) was used to extract genomic DNA from mouse tails and amplify by PCR. The DNA is extracted by incubating the tissue sample with a mixture of the Extraction Solution and the Tissue Preparation Solution. Following addition of Neutralization Solution B, the extracted DNA is combined with primers and the REDExtract-N-Amp PCR Reaction Mix (a 2X reaction mixture containing buffer, salts, dNTPs, Taq polymerase, REDTaq dye, and JumpStart Taq antibody) and is ready for PCR.

#### *2.4.3.1. DNA Extraction from Tissue*

Genomic DNA was extracted from tail clippings (~0.5cm) taken from Gal-1 KO mice. Each tissue sample was placed in an Eppendorf tube into which Extraction solution (50µL) with Tissue Preparation solution (12.5µL) was added and the tubes vortexed. The tissue was then incubated in the solution for 10 mins at RT followed by a

subsequent incubation of three mins at 95°C. In order to halt the reaction Neutralization solution (50µL) was added followed by vortexing of the tubes and tail clippings removed. Prepared DNA solution from tissue extraction is ready to use immediately (alternatively storage at 4°C will maintain its stability for ~ six months).

#### *2.4.3.2. Primer Selection*

A combination of three primers are required to bind to the relevant sequences on single-stranded DNA for performing the PCR to copy those sequences. The name and specific nucleic acid sequence of each of these primers is shown in table 8.

**Table 8. Primers used for genotyping Gal-1 KO mice.**

| Primer | Primer Type | Primer Sequence (5' -> 3') |
|---|---|---|
| oIMR6918 | Common | AAA CTT CAG CCG GGA GAA AGG |
| oIMR6919 | Wild Type Forward | GAC CCC ATC CCT ACA CCC CAG |
| oIMR8306 | Mutant Forward | CTA TCA GGA CAT AGC GTT GG |

#### *2.4.3.3. Gene Extraction and Amplification*

A mastermix of reagents required for the PCR was prepared from REDExtract-N-Amp, the three primers and RNAse free water. The required volumes per sample, shown in table 9, were multiplied by the number of DNA samples to calculate the total volumes needed of each reagent, these volumes were then combined for the mastermix. The mastermix (10µl) was added to PCR tubes containing the DNA solution (2µl) to give a total volume of 12µl per tube. Negative and positive control samples were also prepared using RNAse free water and DNA from a known Gal-1 KO mouse in place of the DNA solutions obtained from the tissue extractions. The PCR tubes of sample

were placed in the PCR machine and thermal cycling was carried out as per manufacturer's guidelines (see table 10).

**Table 9. PCR tube contents for genotyping Gal-1 KO mice.**

| Reagent | Volume (µl) per sample |
|---|---|
| REDExtract-N-Amp | 6 |
| DNA solution from tissue extraction | 2 |
| Common primer (100µM) | 0.12 |
| Wild-type primer(100µM) | 0.12 |
| Mutant primer (100µM) | 0.12 |
| RNAse free water | 3.64 |
| Total | 12 |

**Table 10. Thermal cycling protocol for genotyping Gal-1 KO mice.**

| Step | Temp. (°C) | Time | Note |
|---|---|---|---|
| 1 | 94 | 3 min | - |
| 2 | 94 | 30 sec | Repeat steps 2-4 for 35 cycles |
| 3 | 65 | 1 min | |
| 4 | 72 | 1 min | |
| 5 | 72 | 2 min | - |
| 6 | 10 | - | hold |

*2.4.3.4. DNA Separation*

Following the PCR cycle gel electrophoresis was performed on the samples to separate the DNA. First a 1.5% agarose was prepared by dissolving agarose powder in Tris-acetate EDTA (TAE) buffer with 2µL Gel Red nucleic acid stain added to the solution to allow visualisation of the DNA. The set gel was positioned in an electrophoresis machine and the tank filled with TAE buffer. The amplified DNA (10µL), along with an equal volume of the positive control, negative control and 100bp DNA ladder, was then loaded into the wells.

Due to DNA being negatively charged, samples will run through the gel in a negative to positive direction when a current is applied and DNA of shorter bp length will travel more easily than those of longer bp length. A constant voltage (100V) was passed through the gel until the DNA samples had migrated far enough that the dye front of the lanes had approached the end of the gel.

To visualise the end product size of the samples a UV light box was used. The Ethidium Bromide channel was selected, and the exposure time calculated using the auto-exposure function although generally exposure to ultraviolet illumination for 100ms was the most appropriate. The illuminated bands of the bp ladder were then used as markers to determine the bp length of the bands of DNA from the samples. In this case expected results would be a band indicating DNA of ~390bp for a WT mouse and a band of ~250bp for Gal-1 KO, with both visible for a heterozygous.

### *2.4.4. Western Blotting*

Western blotting is a well-established technique for detection and analysis of proteins. Proteins are separated according to their molecular weight by gel electrophoresis and then undergo electrophoretic transfer to immobilise them at their correct molecular weight on a membrane. Primary antibodies bind to the immobilised protein resulting in formation of a protein:antibody complex. A secondary enzyme conjugated antibody specific for the primary is added and results in the formation of a protein:antibody:antibody:enzyme complex proportional to the amount of protein on the membrane. On addition of the appropriate substrate the enzyme acts to catalyse the reaction resulting in the emission of light and the signal is captured and quantified.

Western blots were performed using the NuPAGE® Bis-Tris Electrophoresis System (Invitrogen). The system operates at a neutral pH which in partnership with the pre-cast, discontinuous SDS-PAGE gels supports the stability of both proteins and gel matrix during electrophoresis.

#### *2.4.4.1. Sample Preparation*

Cell lysate samples were prepared from 2 x $10^6$ neutrophils in 150µl volume in a 1.5ml Eppendorf. Loading buffer was prepared from LDS sample buffer (4X) + DTT (1:100) and heated to 95°C on a heat block. The LDS sample buffer is specifically suited to prepare protein samples to run with Bis-Tris gels. The sample buffer contains lithium dodecyl sulphate and has a slightly alkaline pH which improves activity of the reducing agent, DTT, in cleaving disulphide bonds between cysteine residues and thus

linearising the protein. The Coomassie G250 in the buffer migrates in closely with the ions providing a sharp dye front.

Immediately after the stimulation period, 50µl hot loading buffer was added to neutrophil samples and quickly resuspended before placing in an Eppendorf on the heat block and boiling at 95°C for 5 mins to lyse the samples. Boiled samples were then passed, 10 times, through a 23-gauge needle using a 1ml syringe to shear the DNA and decrease viscosity. Prepared Western blot samples were then stored at -20°C prior to their loading in the gel.

#### *2.4.4.2. Denaturing Gel Electrophoresis*

Gel electrophoresis was performed using a NuPAGE® Novex® Pre-Cast Bis-Tris 12 well 10% acrylamide gel and NuPAGE® MOPS SDS Running Buffer in an XCell SureLock™ Mini-Cell unit. The running buffer, specifically suited for detection of mid-sized proteins on the NuPAGE® Bis-Tris Gels, is a 20X stock solution and was prepared by diluting 25ml stock with 475ml $dH_2O$. To set up the tank the electrode assembly was placed into the tank unit. The 10cm gel cassette was then placed in the tank, flush with the electrode unit and orientated so that the well side of the gel cassette faces inwards towards the buffer core and locked in place with the gel tension wedge. The inner buffer chamber between the two gels was then filled to just above the wells with approximately 200ml running buffer and left for several minutes to check the tightness of the seal. Following confirmation of no leakage the outer chamber of the tank was then filled, until the level surpassed the electrode wire.

An appropriate molecular weight marker was loaded (5µl) in to the first well of the gel, followed by the prepared samples (20µl). The inner buffer chamber was then filled to the top with loading buffer. The voltage was set to 80V for the first ~15mins of running, until the sample had passed through the stacking gel, and then increased to 120V for ~75mins to run the samples to the bottom of the gel.

The electric field applied will have separated proteins by their molecular weight, shape and intrinsic charge. The anion detergents in the loading buffer and running buffer (LDS and SDS respectively) will bind to the protein by disrupting the hydrogen bonds holding it in its native form such that it becomes linearised. The denatured structure, with its polypeptide backbone surrounded by hydrophobic tails provides an overall negative charge. The constant ratio of detergent to protein binding outcomes a uniform mass to charge ratio far more significant than the mass/charge of the protein itself thus allowing it to migrate effectively through the gel when a current is applied, with the smallest proteins travelling furthest. Once gel electrophoresis was complete, as indicated by the sample dye front reaching the end of the gel, the current was stopped, and the electrodes disconnected from the tank.

#### *2.4.4.3. Transfer*

Following the running of the gel the separated proteins were electro-transferred on to a membrane using a wet transfer technique.

The gel cassette was removed from the electrophoresis tank and opened by separating the three bonded sides of the two plates and the shorter back plate carefully lifted off. The stacking gel at the top of the gel was removed and the gel, attached to

the front plate was immersed in transfer buffer (25mM Tris base, 190mM glycine, 10% ethanol, pH 8.3) in the assembly tray.

The blotting cassette was placed black side down in the assembly tray (aside the gel) and the ‘sandwich’ was constructed by building upwards. All layers were of a size larger than the gel but smaller than the blotting cassette and were submerged in transfer buffer prior to their addition. First a thin sponge was sat on the blotting cassette, followed by two layers of filter paper. The gel (with the marker on the right) was then carefully 'slid’ off its front plate on to the filter paper, ensuring no air bubbles were trapped between the layers. The PVDF membrane was activated by immersion in 100% methanol (~30secs) prior to transfer buffer and then placed on top of the gel. Two further layers of filter paper and a piece of sponge followed, after which a roller was used to remove any air bubbles that may have got trapped between the layers and the cassette closed.

The filled cassette was then inserted into the transfer tank with black side of the cassette to black side of tank so that the membrane is closer than the gel to the anode (+). In this way the negatively charged proteins will migrate with the current from the gel towards the positive charge via the membrane, where they will become immobilised and fixed. The electro transfer was run at 80V for 90mins. After 90mins, the current was stopped, the electrodes were disconnected, and the blotting cassette was removed from the transfer tank.

#### *2.4.4.4. Blocking and Antibody Probing*

The membrane was removed from the sandwich and incubated with a blocking solution. To prevent non-specific antibody binding the spaces not already occupied by proteins must be blocked by an agent with greater affinity for the membrane than the antibodies. Here the blocking step was performed using 5% non-fat milk diluted in TBS-T (Tris buffered saline (TBS) (150nM sodium chloride, 2mM Tris base, pH7.4) containing 0.1% Tween) which was incubated with the membrane for 1 hour on a plate rocker (RT).

Antibody probing in Western blots most often involves using a non-labelled primary antibody against the target protein and a species specific labelled secondary antibody. In this way the signal is amplified, and the sensitivity of the assay increased. Following the blocking step membranes were incubated with the target specific primary antibody (for dilution and buffer see table 4) overnight on a plate rocker (4°C). After the incubation period the membrane was washed three times in TBS-T for 10 mins on a plate rocker (RT). The HRP conjugated secondary antibody (see table 4) was then added and incubated with the membrane for 1 hour on a plate rocker (RT). Following incubation, the membrane was washed three times in TBS-T for 10 mins on a plate rocker (RT).

#### *2.4.4.5 Detection and Imaging*

Detection was performed using an enzyme-based chemiluminescent system, a method which requires the addition of a reagent that emits light when it reacts with the enzyme (HRP) conjugated to the secondary antibody. The HRP enzyme catalyses the

oxidation of luminol in the presence of peroxide, a reaction which results in the emission of light.

The chemiluminescent substrate (Immobilon forte western HRP substrate) was incubated briefly (~1min) with the membrane and excess substrate was drained off. The membrane was placed protein side up in an X-ray film developing cassette and transferred to the dark room. A sheet of X-ray film was placed on top of the membrane and the cassette was closed as film was exposed (see table 4). Following the exposure period films were removed from the cassette and fed through a developer machine which contained both the developing and fixing solutions.

#### *2.4.4.6. Analysis*

Densitometry was performed using ImageJ software analysis to provide a value for each band. For quantifying phosphorylation of signalling proteins, the value for the phospho-protein was divided by value of the total protein on an individual sample basis. Normalisation was not performed for Western blots detecting protein levels in cell free culture media.

### *2.5. Statistical Analysis*

Data are expressed as mean ± SEM. Comparisons were analysed for statistical significance. GraphPad Prism (v7 or v8) software was used for all analyses and a $P \leq 0.05$ was considered as statistically significant.

For zymosan peritonitis data each individual time point (2, 6, 24, and 48h, where applicable) leukocyte numbers, apoptosis (percentage of neutrophils in each quadrant), efferocytosis (both percentage and enumeration of cells), plasma cytokine concentrations, BMDM (each phenotype for each marker) and peripheral blood counts were analysed using an unpaired t test. Leukocyte numbers profiled across time were analysed using a two-way repeated measures ANOVA with Sidak's multiple comparisons test.

For K/BxN serum transfer arthritis the histology of the joint (microscopic scoring), lymphocyte numbers and plasma cytokine concentrations were analysed using an unpaired t test. Arthritic scoring indices (macroscopic scoring, paw oedema and weight loss) were analysed using a two-way repeated measures ANOVA with Sidak's multiple comparisons test.

For human neutrophil assays (including each time point for Western blot) data was analysed using a paired t test or a one-way ANOVA with Tukey's multiple comparison as appropriate dependent on the number of groups.

For efferocytosis assays data was analysed using a paired t test or a one-way ANOVA with Tukey's multiple comparison as appropriate dependent on the number of groups.

## Chapter 3: Results

### *3.1. The Role of Endogenous Gal-1 in Acute Self-Resolving Inflammation.*

To assess the role of endogenous Gal-1, a model of zymosan-induced peritonitis was performed in Gal-1 KO mice and their wild-type counterparts. Leukocyte trafficking, apoptosis and clearance were assessed at 2, 6, 24 and 48h post zymosan injection.

#### *3.1.1. Leukocyte Trafficking*

As shown in figure 16A there was a sharp and significant increase in leukocyte influx into the peritoneal cavities of wild-type mice from 2h ($17.59 \times 10^6 \pm 0.51 \times 10^6$) to 6h ($35.01 \times 10^6 \pm 6.32 \times 10^6$) followed by a decline at 24h ($15.50 \times 10^6 \pm 0.54 \times 10^6$). A biphasic response was observed with a second increase in total leukocyte number at 48h ($26.72 \times 10^6 \pm 2.28 \times 10^6$). Leukocyte recruitment in Gal-1 KO mice mirrored that observed in their WT counterparts in terms of the temporal nature of the response, however significantly more leukocytes migrated into the peritoneal cavities of Gal-1 KO mice when compared to WT at the 6h time point ($41.33 \times 10^6 \pm 3.43 \times 10^6$ vs $28.69 \times 10^6 \pm 0.78 \times 10^6$ $p = 0.009$).

Further analysis of leukocyte subtypes identified neutrophils as the predominant cell type during the initiation phase from 2h onwards, with a decline in number observed at 24h (figure 16B). Whilst this trend was observed in both genotypes significantly more neutrophils ($7/4^{+}Ly6G^{high}$) trafficked to the peritoneal cavity of Gal-1 KO mice, compared to WT at the 6h time point ($30.27 \times 10^6 \pm 1.70 \times 10^6$ vs $20.14 \times 10^6 \pm 0.60 \times 10^6$ for WT, $p < 0.0001$, figure 16B).

Monocyte ($7/4^{+}Ly6G^{-}$) numbers displayed a bell shaped trend (figure 16C) with low numbers at 2h, a respective rise at 6 and 24h followed by a decline at 48h ($1.25 \times 10^6 \pm 0.10 \times 10^6$) in WT mice. No difference was observed between genotypes.

Eosinophil (Siglec $F^{+}$) numbers rose sharply at 48h post zymosan in both genotypes (figure 16D), however, no differences in eosinophil number were observed at any of the observed time-points between genotypes.

In WT mice, macrophage ($F4/80^{+}$) numbers increased with time from 2h to 6h, remain unchanged at 24h before rising sharply at 48h (figure 16E). At early time points there was no significant difference between macrophage numbers in the cavities of Gal-1 KO mice compared to WT, however significantly more macrophages were present in Gal-1 KO mice at 48h ($20.98 \times 10^6 \pm 1.02 \times 10^6$ vs $17.86 \times 10^6 \pm 1.13 \times 10^6$ for WT, $p = 0.011$, figure 16E). Macrophage phenotype was also assessed. There were no significant differences between genotypes in the number of mature macrophages ($F4/80^{+}CD11b^{high}$), however significantly more resolving macrophages ($F4/80^{+}CD11b^{low}$) were present within the cavities of Gal-1 KO mice at 48h compared to their WT counterparts ($11.89 \times 10^6 \pm 0.84 \times 10^6$ vs $9.08 \times 10^6 \pm 0.89 \times 10^6$, $P = 0.003$, figures 16F & 16G).

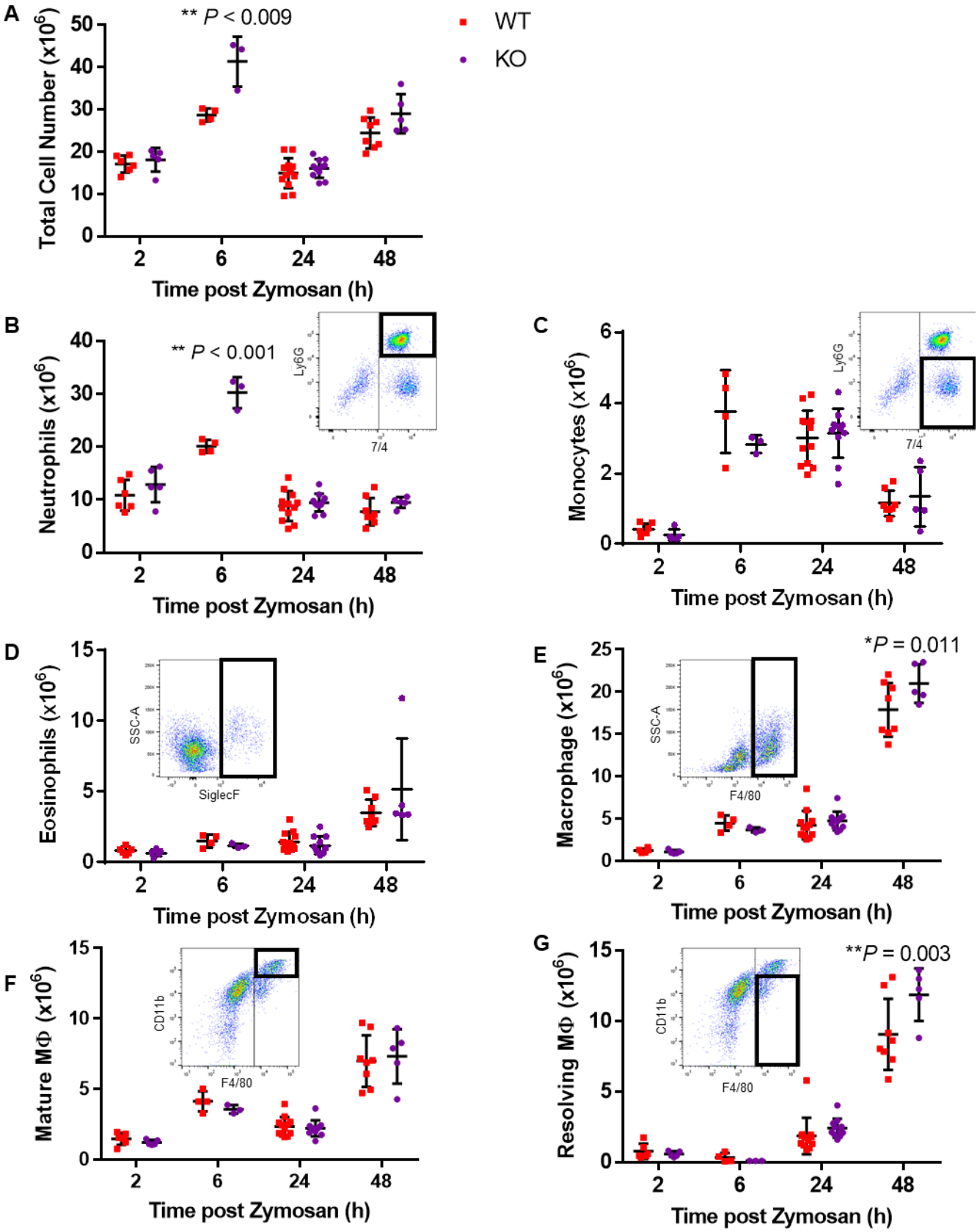

A
** P < 0.009
WT
KO
Total Cell Number (x10^6)
Time post Zymosan (h)
B
** P < 0.001
Neutrophils (x10^6)
Ly6G
7/4
C
Monocytes (x10^6)
D
Eosinophils (x10^6)
SSC-A
SiglecF
E
*P = 0.011
Macrophage (x10^6)
F4/80
F
Mature MΦ (x10^6)
CD11b
G
**P = 0.003
Resolving MΦ (x10^6)

**Figure 16. Leukocyte recruitment is enhanced in Gal-1 KO mice.**
Mice received zymosan (1mg i.p.) and peritoneal lavage was performed at 2h, 6h, 24h and 48h. Total cell counts were performed (**A**) and the number of neutrophils (7/4$^{+}$Ly6G$^{+}$) (**B**), inflammatory monocytes (7/4$^{+}$Ly6G$^{-}$) (**C**), eosinophils (Siglec F$^{+}$) (**D**), macrophages (F4/80$^{+}$) (**E**), mature macrophages (F4/80$^{+}$CD11b$^{high}$) (**F**) and resolving macrophages (F4/80$^{+}$CD11b$^{low}$) (**G**) were identified by flow cytometry. Representative flow cytometry plots are shown for each subset. Statistical analysis was performed using a two-way ANOVA with Sidak's multiple comparisons test, results are displayed as mean ± SEM, in all cases significant results are considered as $P < 0.05$. n=3-12.

### *3.1.2. Neutrophil Apoptosis*

Neutrophils ($Ly6G^+$ cells) that were collected from the peritoneal cavity were assessed for apoptosis using Annexin V (AnxV) and the viability dye Zombie NIR (figure 17A). At 2h post zymosan most neutrophils from within the cavity were viable ($AnxV^-NIR^-$; 78.79% ± 0.49%. figure 17B) with the majority of the remaining neutrophils being early apoptotic ($AnxV^+NIR^-$; 16.25% ± 0.21%). Very few neutrophils were late apoptotic ($AnxV^+NIR^+$) or necrotic ($AnxV^-NIR^+$; 3.89% ± 0.12% or 1.08% ± 0.17% respectively). No significant differences in the percentage of apoptotic neutrophils were observed between WT and Gal-1 KO mice. At 6h post-zymosan (figure 17C), the majority of neutrophils (72.25% ± 0.45%) were early apoptotic with few cells in late apoptosis (3.09% ± 0.92%) or necrosis (0.07% ± 0.04%). Again, there were no significant differences between cell states of WT and Gal-1 KO mice. At 24h post zymosan the remaining neutrophils were mainly viable (72.36% ± 5.41%) with some early apoptotic (22.70% ± 4.32%) and late apoptotic cells detectable (4.56% ± 0.90%, figure 17D). As observed at earlier time-points there were no significant differences between WT and Gal-1 KO cells. When assessed 48h after administration of zymosan, neutrophils were predominantly viable (69.96% ± 2.72%). Again, no differences were observed between genotypes (figure 17E).

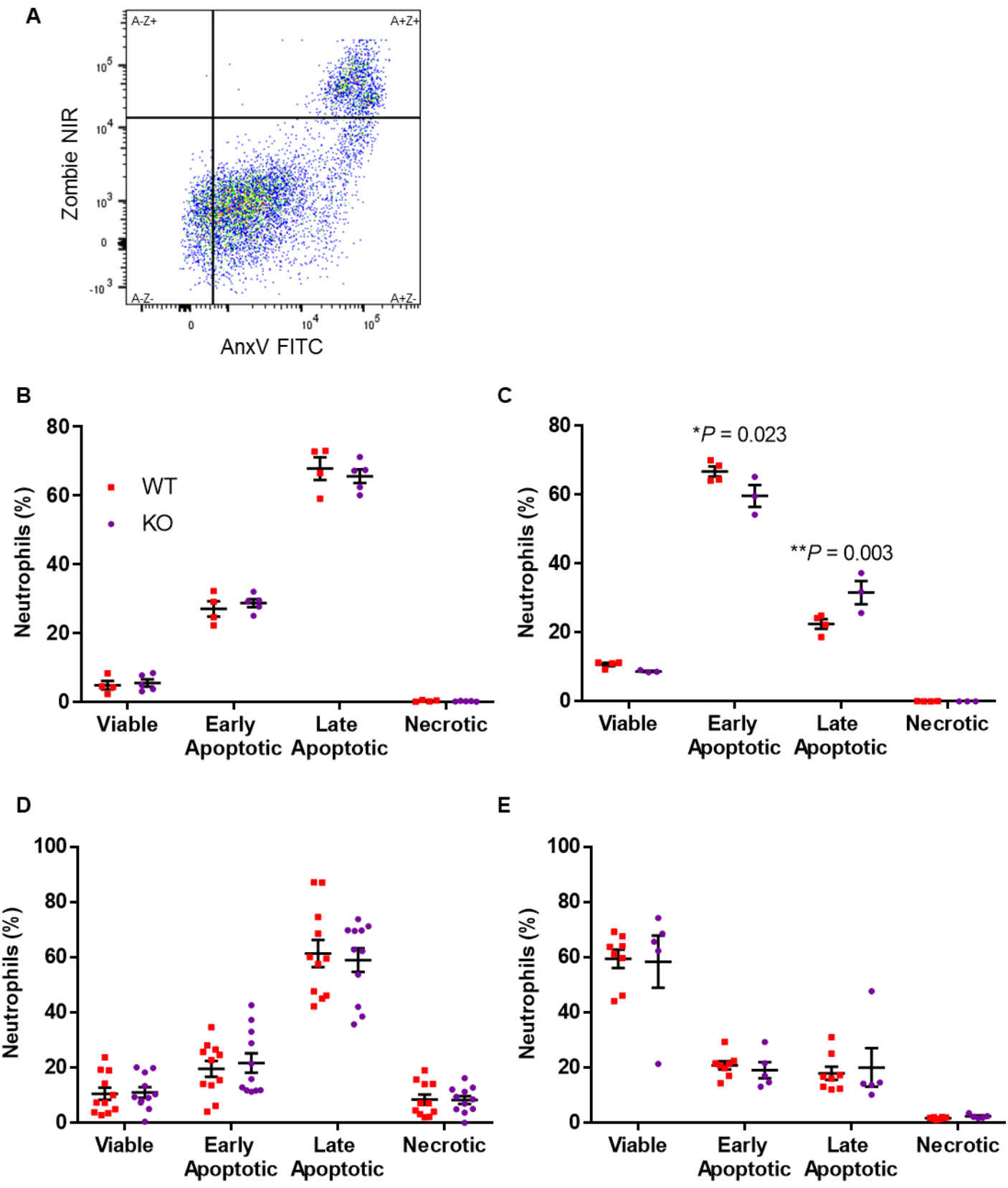

A
A-Z+
A+Z+
A-Z-
A+Z-
Zombie NIR
AnxV FITC
B
C
D
E
Neutrophils (%)
WT
KO
*P = 0.023
**P = 0.003
Viable
Early Apoptotic
Late Apoptotic
Necrotic

**Figure 17. *In vivo* neutrophil apoptosis is not effected by the absence of Gal-1.**
Mice received zymosan (1mg i.p.) and peritoneal lavage was performed at 2h, 6h, 24h and 48h. Flow cytometry was used to select the neutrophil ($Ly6G^+$) population, which was further assessed by AnxV binding and Zombie (NIR) staining. A quadrant gating strategy was applied to determine (**A**) viable ($AnxV^-NIR^-$), early apoptotic ($AnxV^+NIR^-$), late apoptotic ($AnxV^+NIR^+$) and necrotic ($AnxV^-NIR^+$) neutrophil populations. Results for the percentages of the neutrophil populations within each of the quadrants is shown for 2h (**B**), 6h (**C**), 24h (**D**) and 48h (**E**). Statistical analysis was performed using an unpaired t-test for each quadrant at each time point with results displayed showing the mean ± SEM, in all cases significant results are considered as $P < 0.05$. n = 3-12.

As apoptotic neutrophils are rapidly cleared from the peritoneum *in vivo*, cells from peritoneal exudates were collected and incubated overnight to assess *ex vivo* apoptosis. Analysis of neutrophil apoptosis *ex vivo* was performed at 2, 6, 24 and 48h post injection of zymosan using AnxV and Zombie NIR (figure 18A).

Neutrophils from the peritoneal exudate of WT mice collected 2h post zymosan and assessed for apoptosis following overnight incubation were predominantly apoptotic with a large population of late apoptotic (66.77% ± 1.1%) and a smaller population of early apoptotic (27.84% ± 0.84%) cells observed. A small viable population of neutrophils were detectable (5.19% ± 0.34%) as well as a negligible population of cells shown to be necrotic (0.214% ± 0.05%) (figure 18B). There were no significant differences between genotypes in any of the populations of neutrophils.

When neutrophils were collected from the peritoneal cavity at 6h post zymosan injection and incubated *ex vivo*, the majority of cells were early apoptotic (63.21% ± 3.54%; figure 18C) with a considerable population of late apoptotic cells (27.05% ± 4.55%). Neutrophils from Gal-1 KO mice were further along the apoptotic pathway than cells from WT mice, with significantly higher numbers of late apoptotic neutrophils in cultures from Gal-1 KO mice compared to WT (31.6% ± 3.35% vs 22.5% ± 1.39%; $p = 0.003$).

No significant differences in apoptosis were observed between genotypes when neutrophils were harvested from the peritoneal cavity at either 24 or 48h post-zymosan (figure 18D & E).

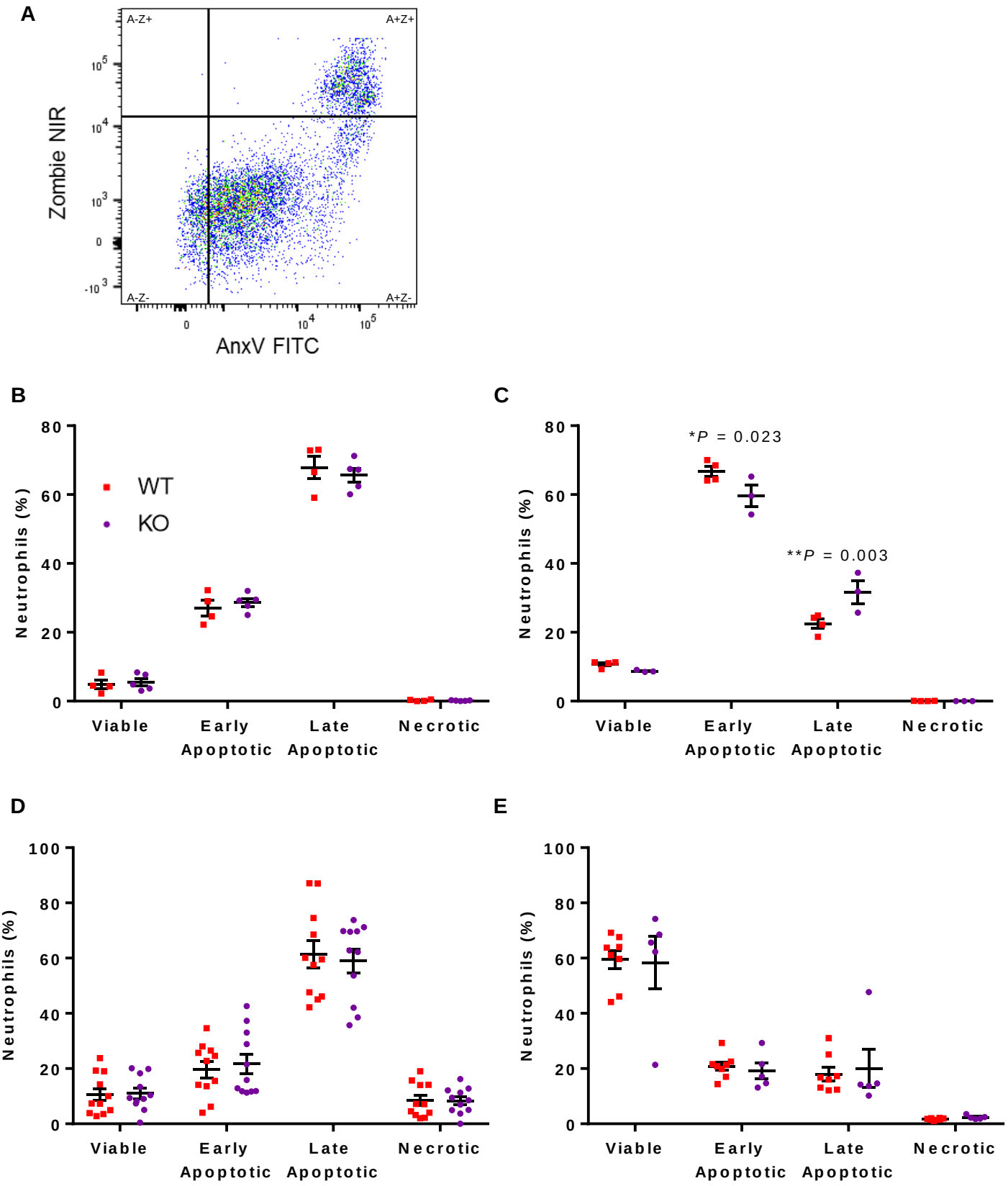

A
A-Z+
A+Z+
A-Z-
A+Z-
Zombie NIR
AnxV FITC
B
C
WT
KO
Neutrophils (%)
*P = 0.023
**P = 0.003
Viable
Early Apoptotic
Late Apoptotic
Necrotic
D
E

**Figure 18. Gal-1 KO neutrophils from peak inflammation display enhanced apoptosis *ex vivo.***
Mice received zymosan (1mg i.p.) and peritoneal lavage was performed at 2h, 6h, 24h and 48h. Cells from 1ml of peritoneal exudate were resuspended in RPMI + 0.5% BSA and incubated for 20h. Flow cytometry was used to select the neutrophil ($Ly6G^+$) population, which was further assessed by AnxV binding and Zombie (NIR) staining. A quadrant gating strategy (**A**) was applied to determine viable ($AnxV^-NIR^-$), early apoptotic ($AnxV^+NIR^-$), late apoptotic ($AnxV^+NIR^+$) and necrotic ($AnxV^-NIR^+$) neutrophil populations. Results for the percentages of the neutrophil population within each of the quadrants is shown for 2h (**B**), 6h (**C**), 24h (**D**) and 48h (**E**). Statistical analysis was performed using an unpaired t-test for each quadrant at each time point with results displayed showing the mean ± SEM, in all cases significant results are considered as $P < 0.05$. n = 3-12.

### *3.1.3. Neutrophil Clearance*

At 24h, significantly more macrophages had efferocytosed neutrophils in Gal-1 KO mice compared to WT (5.14% ± 0.36% vs 3.92% ± 0.26%; p = 0.011, figure 19A). However, further analysis indicated a significantly lower MFI in the Ly6G channel of Gal-1 KO macrophages indicating that fewer neutrophils had been efferocytosed per macrophage compared to WT (0.86 $x10^4$ ± 0.14 $x10^4$ vs 1.27 $x10^4$ ± 0.13 $x10^4$; p = 0.009; figure 19B). At 48h there were no significant difference in either parameter between genotypes (figures 19C & 19D).

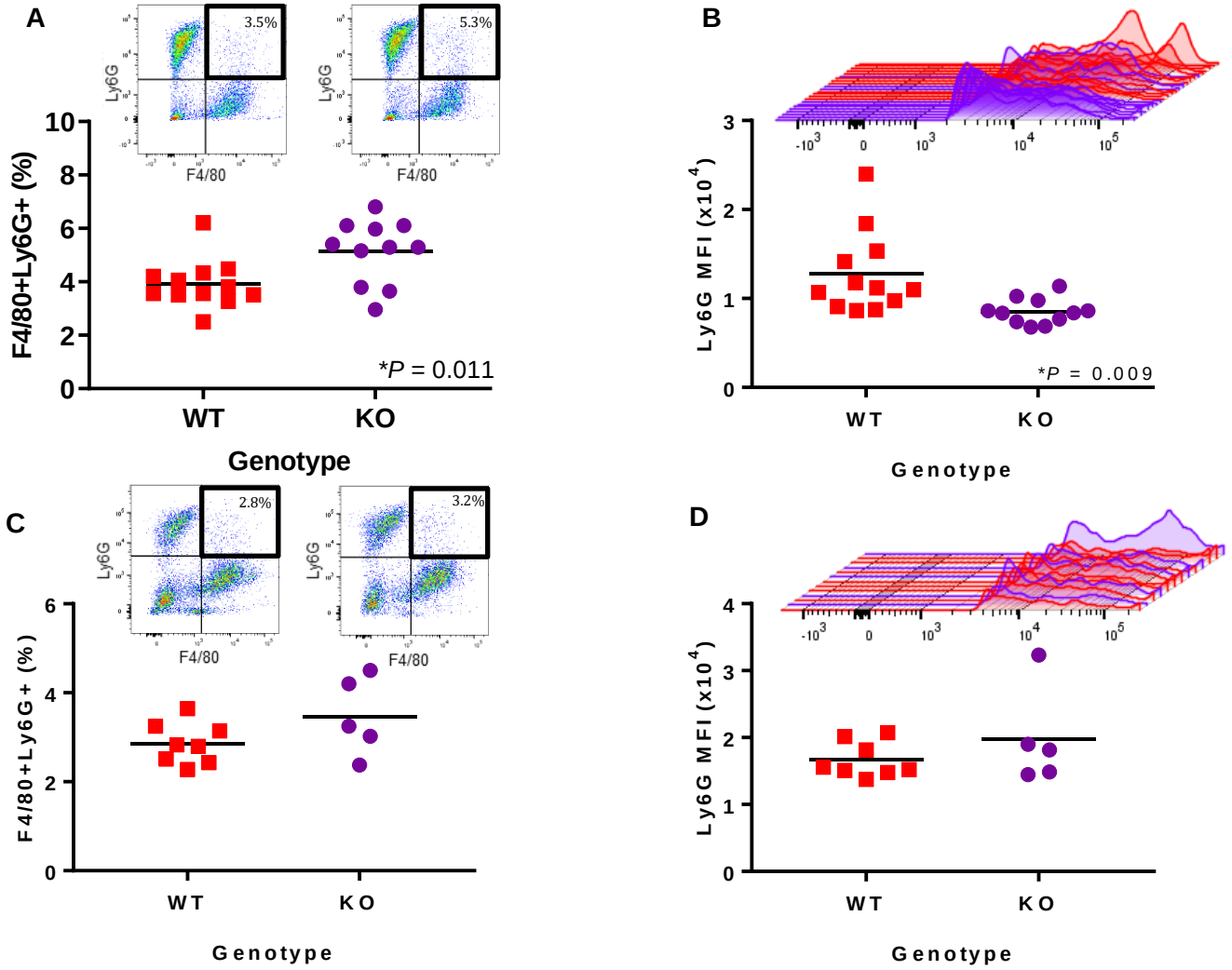



**Figure 19. *In vivo* efferocytosis is modulated in the absence of endogenous Gal-1.**
Mice received zymosan (1mg i.p.) and peritoneal lavage was performed at 24h and 48h. Cell surface staining for macrophages (F4/80$^+$) was followed by fixation, permeabilisation and neutrophil (Ly6G$^+$) staining. A quadrant gating strategy was applied to determine the percentage of cells positive for efferocytosis (F4/80$^+$Ly6G$^+$) with representative plots for both genotypes inlaid. Results for the percentage of cells positive for efferocytosis at 24h (**A**) and 48h (**C**) are displayed with the mean for both genotypes. The median fluorescence intensity (MFI) for the neutrophil (Ly6G$^+$) staining of double positive (F4/80$^+$Ly6G$^+$) cells was also analysed to compare the relative amount of neutrophils within macrophages at 24h (**B**) and 48h (**D**) and representative MFI values shown. Statistical analysis was performed using an unpaired t-test and significant results considered as $P < 0.05$. n = 5-12.

### *3.1.4. Inflammatory Mediator Profile*

Peritoneal lavage fluid was collected at 2h post zymosan and cell free exudates analysed by Luminex (Labospace Ltd. Milan) for levels of key inflammatory proteins. This data revealed a trend towards increased levels of chemokines CCL2 (MCP-1), CCL3 (MIP-1α) and CXCL1 (KC) (shown in figures 20A-C), the pro-inflammatory cytokine TNF-α (figures 20D) and the growth factor granulocyte macrophage colony stimulating factor (GM-CSF) (figures 20G) with roles in cell survival and trafficking were all elevated in Gal-1 KO mice. Significantly elevated levels of IL-6 (figure 20F) and VEGF (figure 20H) were detected in the lavage fluid of Gal-1 KO mice compared to WT mice, whereas the levels of Il-1β were similar for both genotypes (figure 20E).

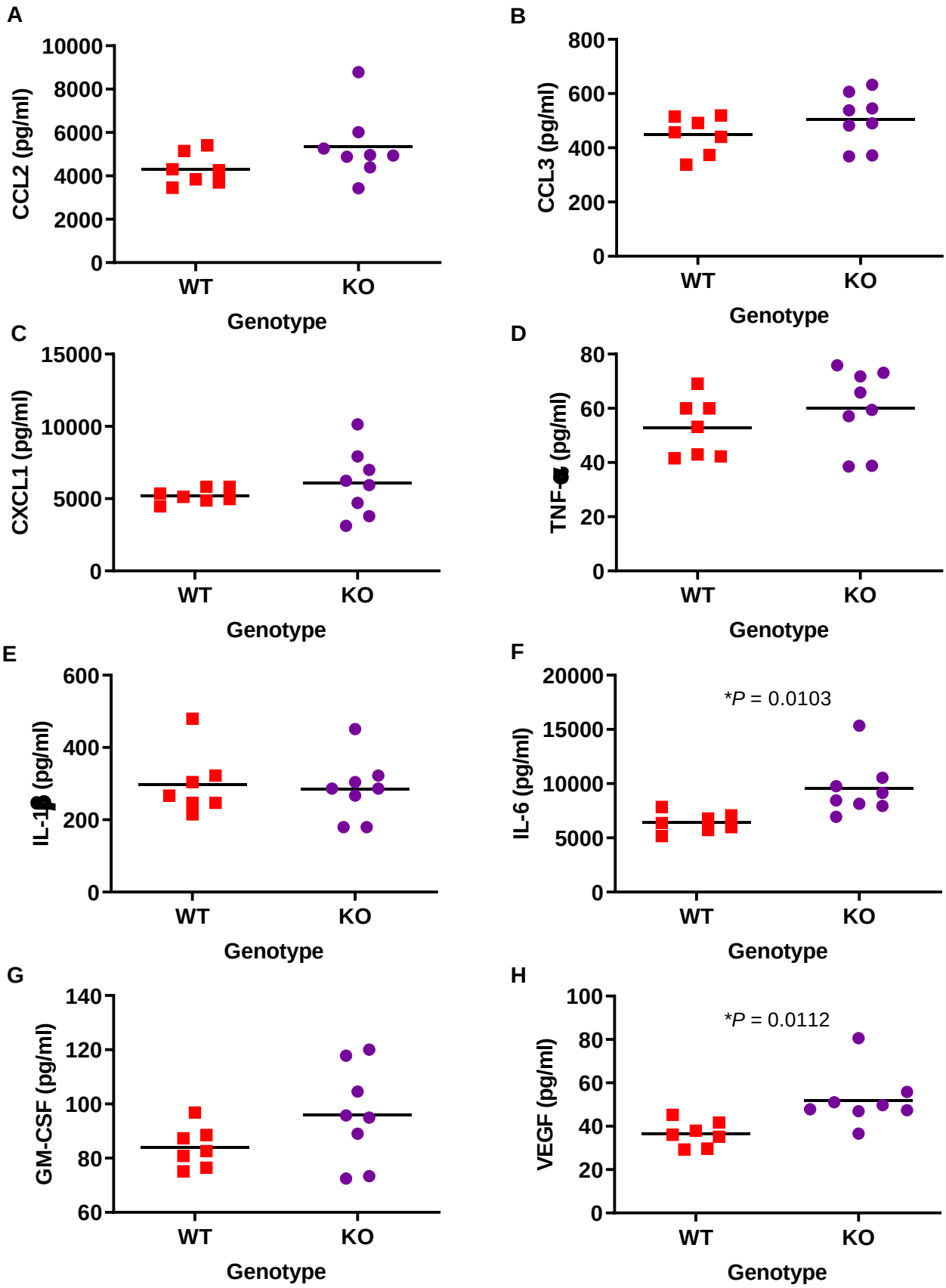

A
CCL2 (pg/ml)
10000
8000
6000
4000
2000
0
WT
KO
Genotype
B
CCL3 (pg/ml)
800
600
400
200
0
WT
KO
Genotype
C
CXCL1 (pg/ml)
15000
10000
5000
0
WT
KO
Genotype
D
TNF-α (pg/ml)
80
60
40
20
0
WT
KO
Genotype
E
IL-1β (pg/ml)
600
400
200
0
WT
KO
Genotype
F
*P = 0.0103
IL-6 (pg/ml)
20000
15000
10000
5000
0
WT
KO
Genotype
G
GM-CSF (pg/ml)
140
120
100
80
60
WT
KO
Genotype
H
*P = 0.0112
VEGF (pg/ml)
100
80
60
40
20
0
WT
KO
Genotype

**Figure 20. Inflammatory mediators are increased in Gal-1 KO mice**

The supernatant was collected from 1ml of peritoneal exudate (2h zymosan peritonitis) and concentrations of analytes measured by luminex. Levels of chemokines (**A**) CCL2 (MCP-1), (**B**) CCL3 (MIP-1-α) and (**C**) CXCL-1 (KC) and inflammatory cytokines (**D**) TNF-α, (**E**) IL-1β and (**F**) IL-6 are shown for both genotypes, as well as the growth factors (**G**) GM-CSF and (**H**) VEGF. Statistical analysis was performed using an unpaired t-test and significant results considered as $P < 0.05$. n=7-8.

### *3.1.5. Macrophage Phenotype Skewing*

Bone Marrow Derived Macrophages (BMDM) from WT and Gal-1 KO mice were skewed using cytokines and endotoxin and macrophage M1 markers (CD86 and MHC-II) and M2 markers (CD206, CD36 and MerTK) were analysed. Macrophages from Gal-1 KO mice had significantly lower CD86 ($p = 0.003$) and significantly higher CD206 ($p = 0.017$) on the MO and M2 populations compared to WT mice, indicative or a more M2-like phenotype (figure 21A & B). Additionally, Gal-1 KO macrophages had higher surface expression of the scavenger receptors CD36 (figure 21C) and MerTK (figure 21D) compared to WT, indicative of an M2-like phenotype. Somewhat surprisingly, MHC-II expression, which is associated with M1-like macrophages was also slightly higher on Gal-1 KO macrophages when compared to WT cells (as shown in figure 21E).

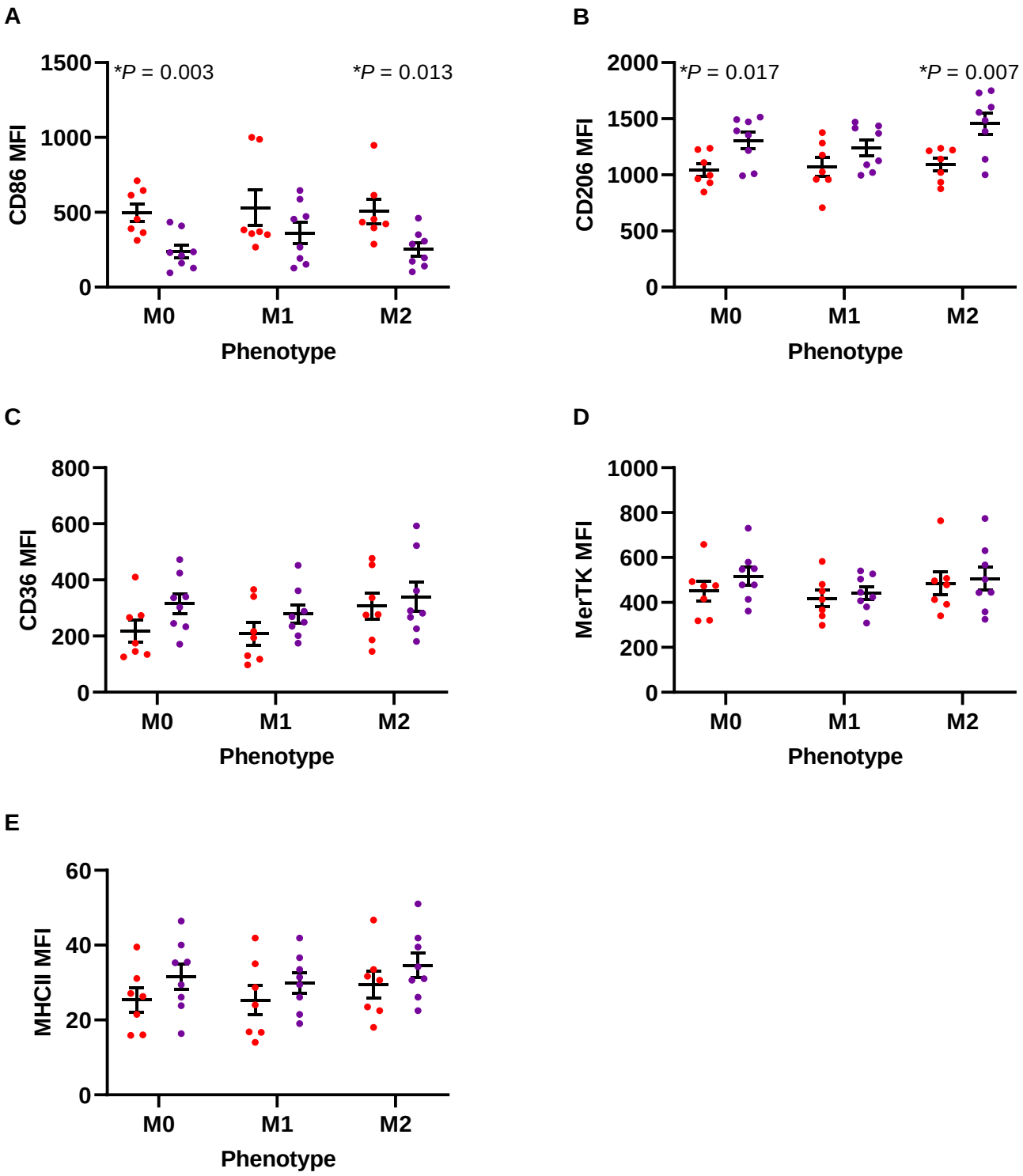



**Figure 21. Bone Marrow Derived Macrophages from Gal-1 KO mice exhibit a more M2-like phenotype.** Mice (WT or Gal-1 KO) were sacrificed 2h post zymosan (1mg i.p.) and bone marrow cells were collected from the femur and differentiated to macrophages. BMDM were skewed with M1 (10ng/ml LPS + 20ng/ml IFNγ) / M2 (20ng/ml IL-4) mediators for 3 days. Macrophage phenotyping was performed by flow cytometry to detect surface expression of CD86 (**A**), CD206 (**B**), CD36 (**C**), MerTK (**D**) and MHC-II (**E**). Statistical analysis was performed using an unpaired t-test for each phenotype for each marker and significant results considered as $P < 0.05$. n=7-8 mice per group.

### *3.2. The Effect of Exogenous Gal-1 on the Resolution of Acute Inflammation.*

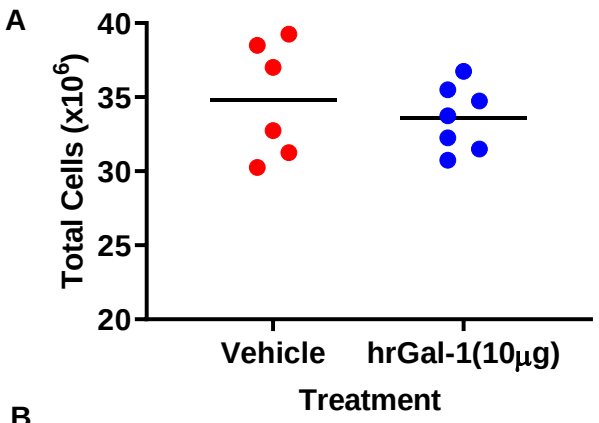
Given the enhanced neutrophil recruitment observed in Gal-1 KO mice, whether exogenous human recombinant Gal-1 can modulate neutrophil trafficking and clearance to drive inflammatory resolution was next assessed. To this end, hrGal-1 was administered either during the recruitment phase at 2h post-zymosan (and lavage performed at 6h), or at the peak; 8h post-zymosan (and lavage performed at 24 and 48h).

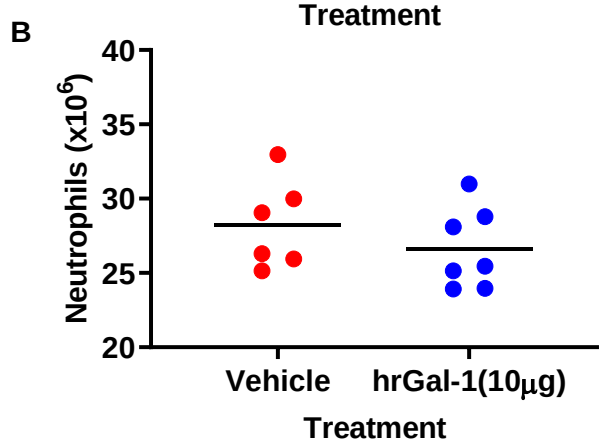
#### *3.2.1. Leukocyte Trafficking*

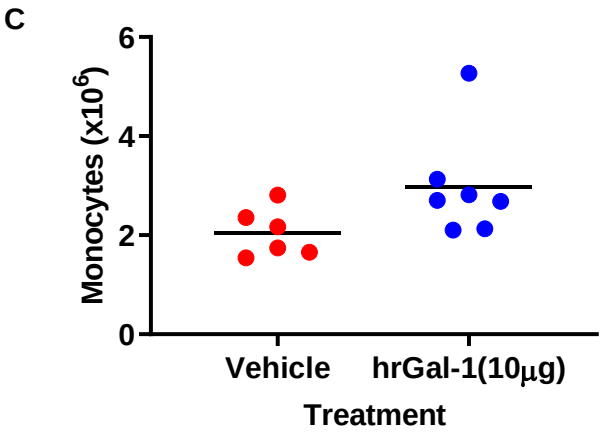
Administration of hrGal-1 (10ug) at 2h post zymosan had no significant effect on leukocyte recruitment to the peritoneum as assessed at 6h post zymosan (figure 22A-G).

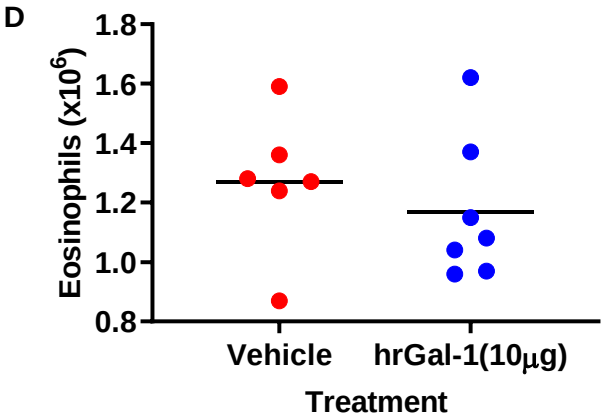
To assess resolution hrGal-1 was administered at peak (8h) and lavage performed at 24h and 48h post zymosan. Administration of hrGal-1 at this time-point resulted in significantly fewer leukocytes being recovered 16h later (24h time point) compared to those recovered from vehicle treated mice (figure 23A). As shown in figures 23B-G further analysis of the individual leukocyte subtypes indicated that there was a trend towards an all-round decrease in the numbers of all leukocyte subsets analysed in the cavities of mice treated with hrGal-1. Significant reductions in the numbers of neutrophils ($7/4^{+}Ly6G^{+}$; figure 23B), eosinophils (SiglecF+; figure 23D) and resolving macrophages ($F4/80+CD11b^{low}$; figure 23G) were observed. At the 48h timepoint significantly fewer leukocytes were recovered from the peritoneal cavities of mice treated with hrGal-1 compared to vehicle treated mice ($7.05 \times 10^6 \pm 1.28 \times 10^6$ vs 12.90

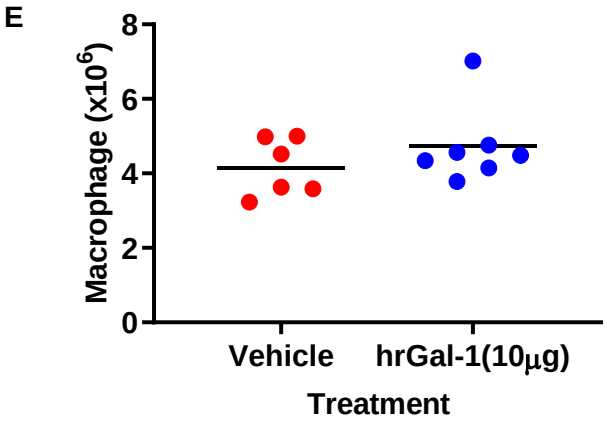
x $10^6$ ± 2.50 x $10^{6;}$ $p < 0.037$, table 11). Further analysis of leukocyte subtypes indicated a trend towards decreased neutrophils numbers and significantly fewer monocytes ($7/4^+Ly6G^-$), total macrophages ($F4/80^+$) and mature macrophages ($F4/80^+CD11b^{high}$) in the cavities of mice treated with hrGal-1 at the 48h time point (table 11).

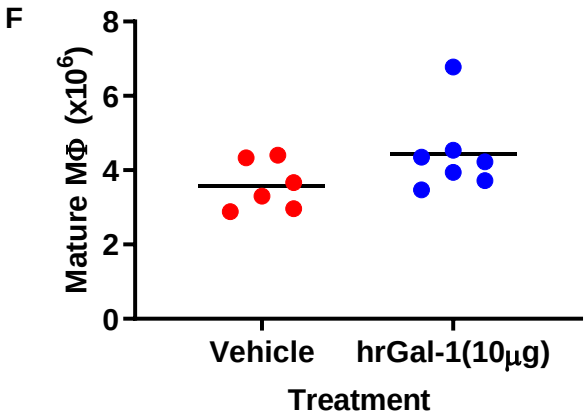

A
Total Cells (x10^6)
40
35
30
25
20
Vehicle
hrGal-1(10μg)
Treatment


B
Neutrophils (x10^6)
40
35
30
25
20
Vehicle
hrGal-1(10μg)
Treatment


C
Monocytes (x10^6)
6
4
2
0
Vehicle
hrGal-1(10μg)
Treatment


D
Eosinophils (x10^6)
1.8
1.6
1.4
1.2
1.0
0.8
Vehicle
hrGal-1(10μg)
Treatment


E
Macrophage (x10^6)
8
6
4
2
0
Vehicle
hrGal-1(10μg)
Treatment


F
Mature MΦ (x10^6)
8
6
4
2
0
Vehicle
hrGal-1(10μg)
Treatment

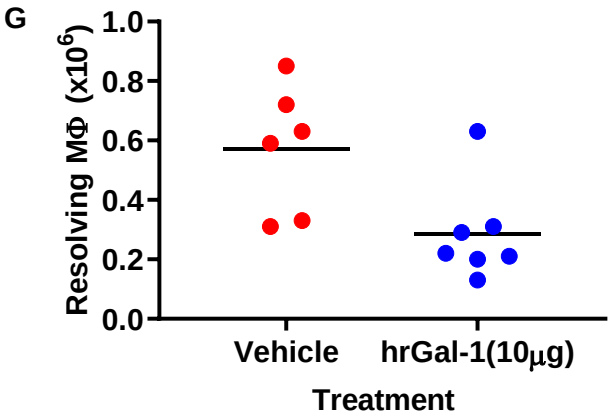
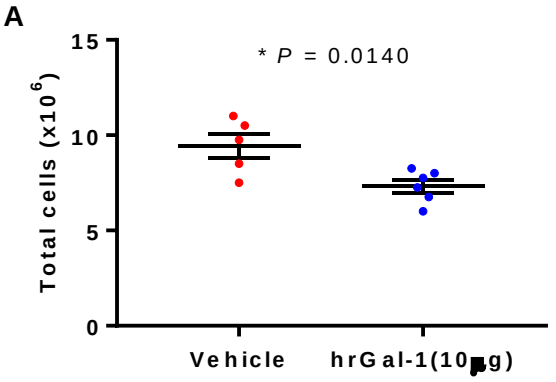
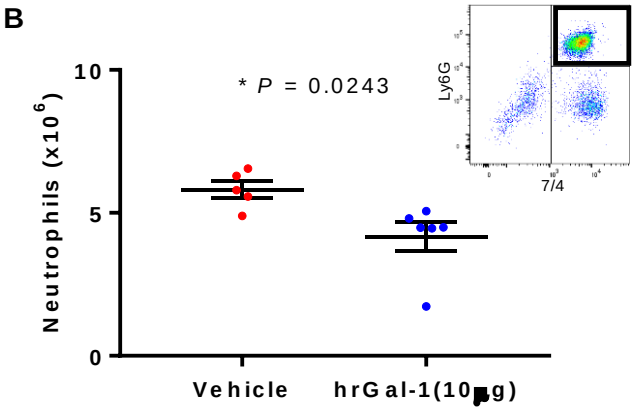
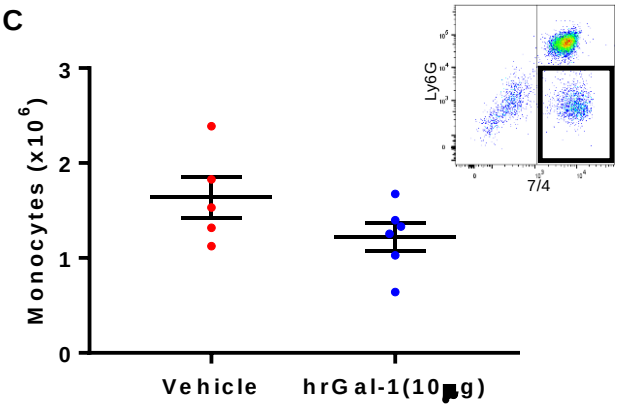
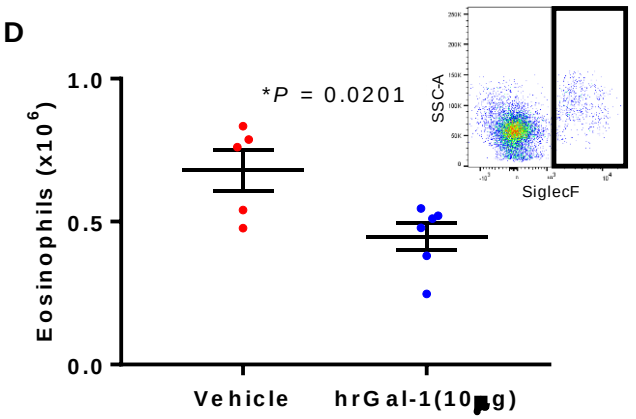

G
Resolving MΦ (x10^6)
1.0
0.8
0.6
0.4
0.2
0.0
Vehicle
hrGal-1(10μg)
Treatment

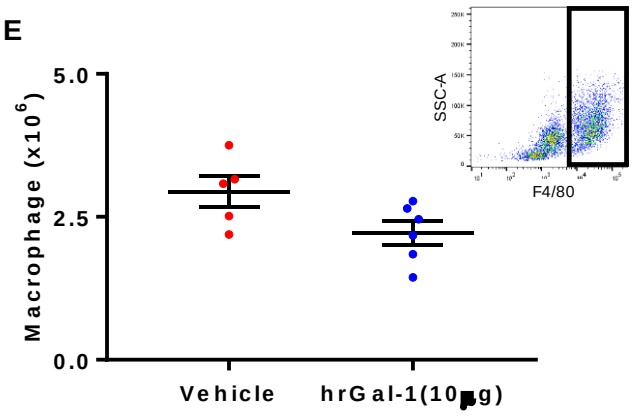
**Figure 22. Administration of exogenous Gal-1 during the early phase of neutrophil infiltration does not modulate leukocyte recruitment.**
Mice received zymosan (1mg i.p.) and were administered hrGal-1 (10µg) or vehicle (200µl $DPBS^{-/-}$) 2h later during the early phase of neutrophil infiltration. Peritoneal lavage was performed at 6h and cells from the exudate were counted to enumerate total cells ($CD45^{+}$) (**A**). Neutrophils ($7/4^{+}Ly6G^{+}$) (**B**), inflammatory monocytes ($7/4^{+}Ly6G^{-}$) (**C**), eosinophils (Siglec $F^{+}$) (**D**), macrophages ($F4/80^{+}$) (**E**), mature macrophages ($F4/80^{+}CD11b^{high}$) (**F**) and resolving macrophages ($F4/80^{+}CD11b^{low}$) (**G**) were determined using flow cytometry. Statistical analysis was performed using an unpaired t-test with results displayed showing the mean ± SEM, in all cases significant results are considered as $P < 0.05$. n=6-7.

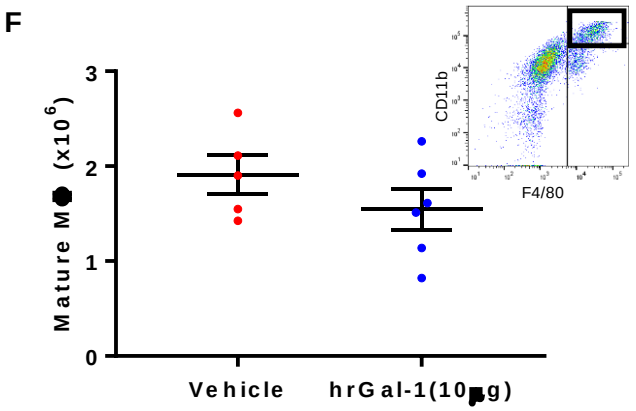

A
* P = 0.0140
Total cells (x10^6)
15
10
5
0
Vehicle
hrGal-1(10µg)


B
* P = 0.0243
Neutrophils (x10^6)
10
5
0
Ly6G
7/4
Vehicle
hrGal-1(10µg)


C
Monocytes (x10^6)
3
2
1
0
Ly6G
7/4
Vehicle
hrGal-1(10µg)


D
*P = 0.0201
Eosinophils (x10^6)
1.0
0.5
0.0
SSC-A
SiglecF
Vehicle
hrGal-1(10µg)


E
Macrophage (x10^6)
5.0
2.5
0.0
SSC-A
F4/80
Vehicle
hrGal-1(10µg)


F
Mature M⬢ (x10^6)
3
2
1
0
CD11b
F4/80
Vehicle
hrGal-1(10µg)

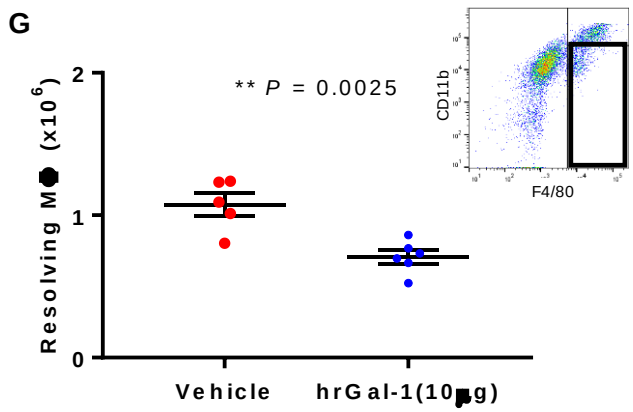

G
** P = 0.0025
Resolving M⬢ (x10^6)
2
1
0
CD11b
F4/80
Vehicle
hrGal-1(10µg)

**Figure 23. Exogenous Gal-1 administraton during the peak of inflammation promotes neutrophil clearance.** Mice received zymosan (1mg i.p.) and were administered hrGal-1 (10µg) or vehicle (200µl DPBS$^{-/-}$) 8h later during the peak of neutrophil infiltration. Peritoneal lavage was performed at 24h Cells from the exudate were counted to enumerate total cells (CD45$^{+}$) (**A**) and neutrophils (7/4$^{+}$Ly6G$^{+}$) (**B**), inflammatory monocytes (7/4$^{+}$Ly6G$^{-}$) (**C**), eosinophils (Siglec F$^{+}$) (**D**), macrophages (F4/80$^{+}$) (**E**), mature macrophages (F4/80$^{+}$CD11b$^{high}$) (**F**) and resolving macrophages (F4/80$^{+}$CD11b$^{low}$) (**G**) were determined using flow cytometry. Representative flow cytometry gating plots are inset for each subset. Statistical analysis was performed using a two-way ANOVA with Sidak's multiple comparisons test, results display the mean ± SEM, in all cases significant results are considered as $P < 0.05$. n=5-6.

## Table 11. Leukocyte numbers at 48h following exogenous Gal-1 during the peak.

| Leukocytes (x $10^6$) | **Vehicle** | **hrGal-1 (10µg)** | **P value** |
|---|---|---|---|
| **Total Cells** | 12.9 ± 2.50 | 7.05 ± 1.28 | 0.0708 |
| **Neutrophils** | 4.38 ± 1.23 | 2.91 ± 0.38 | 0.2834 |
| **Monocytes** | 2.17 ± 0.57 | 0.98 ± 0.18 | 0.0806 |
| **Macrophages** | 5.33 ± 1.57 | 2.32 ± 0.46 | 0.0975 |
| **Mature Macrophages** | 3.90 ± 1.26 | 1.43 ± 0.26 | 0.0904 |
| **Resolving Macrophages** | 1.45 ± 0.32 | 1.05 ± 0.23 | 0.3409 |
| **Eosinophils** | 2.12 ± 0.55 | 1.44 ± 0.14 | 0.2635 |

In order to quantitate the pro-resolving actions of Gal-1, resolution indices were calculated. These are widely utilized to identify mediators that stimulate, as well as those that disrupt or delay resolution (Bannenberg *et al.*, 2005; Schwab *et al.*, 2007; Navarro-Xavier *et al.*, 2010). Figure 24 shows remission indices in response to exogenous Gal-1 based on the neutrophil counts for hrGal-1 treated mice compared to those receiving vehicle. Values calculated indicate the maximum neutrophil number ($T_{max}$), when 50% of this maximum was reached ($T_{50}$) and the resolution interval ($R_i$; time from $T_{max}$ to $T_{50}$). Treatment with hrGal-1 reduced the resolution interval from 39h observed in vehicle treated mice to 14h (figure 24) indicating that neutrophils are cleared faster from the peritoneal cavity as a result of administration of Gal-1.

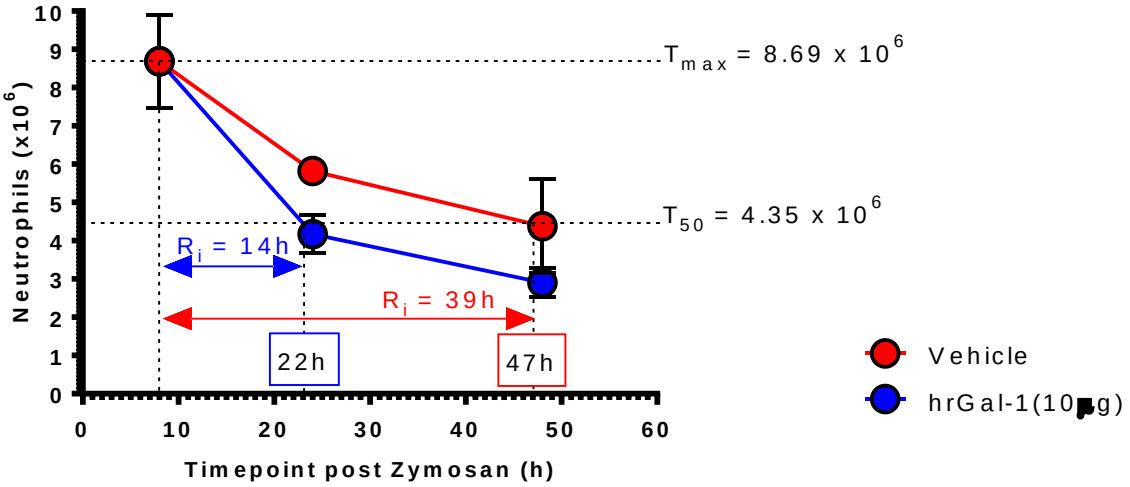



**Figure 24. Exogenous Gal-1 reduced the resolution interval.**
Mice received zymosan (1mg i.p.) and were administered hrGal-1 (10µg) or vehicle (200µl $DPBS^{-/-}$) 8h later during the peak of neutrophil infiltration. Peritoneal lavage was performed at 8h for baseline and subsequently at 24h and 48h. Total cell counts were performed, and flow cytometry was used to quantify neutrophils ($7/4^{+}Ly6G^{+}$). Remission indices were calculated to indicate when mice reached the maximum neutrophil number ($T_{max}$), when 50% of this maximum was reached ($T_{50}$) and the resolution interval ($R_i$) the time from $T_{max}$ to $T_{50}$. n=5-6.

### *3.2.2. Neutrophil Apoptosis*

Analysis of *in vivo* neutrophil apoptosis was performed at 6h (with hrGal-1 given at 2h), as well as at 24 and 48h (with hrGal-1 given at 8h) post injection of zymosan with AnxV and Zombie NIR (figure 25A).

At the 6h time point (figure 25B), neutrophils were predominantly viable (78.92% ± 1.70%) with most others being either early apoptotic (16.32% ± 1.17%), late apoptotic (4.57% ± 0.56%) or necrotic (0.19% ± 0.01%). Significantly fewer viable cells were evident at the 6h time point in mice treated with hrGal-1 when compared to vehicle (77.21% ± 1.3% vs 80.62% ± 1.51%; p= 0.049), with a trend for increased early and late apoptotic neutrophils with hrGal-1 treatment.

At 24h post zymosan significantly fewer viable cells were detected within the peritoneal cavities of mice treated with hrGal-1 compared to vehicle (65.48% ± 3.97% vs 75.9% ± 2.34%; p = 0.018; figure 25C) and a significant increase in the percentage of early apoptotic neutrophils ($AnxV^+/PI^-$) was observed. In contrast, 48h after administration of zymosan significantly more viable cells were observed in the peritoneal cavities of mice that received hrGal-1 compared to vehicle (figure 25D; 79.00% ± 0.061% vs 53.46% ± 7.83%; p = 0.0001).

The percentage of late apoptotic cells was minimal in both genotypes at all time points with negligible numbers of necrotic neutrophils detected.

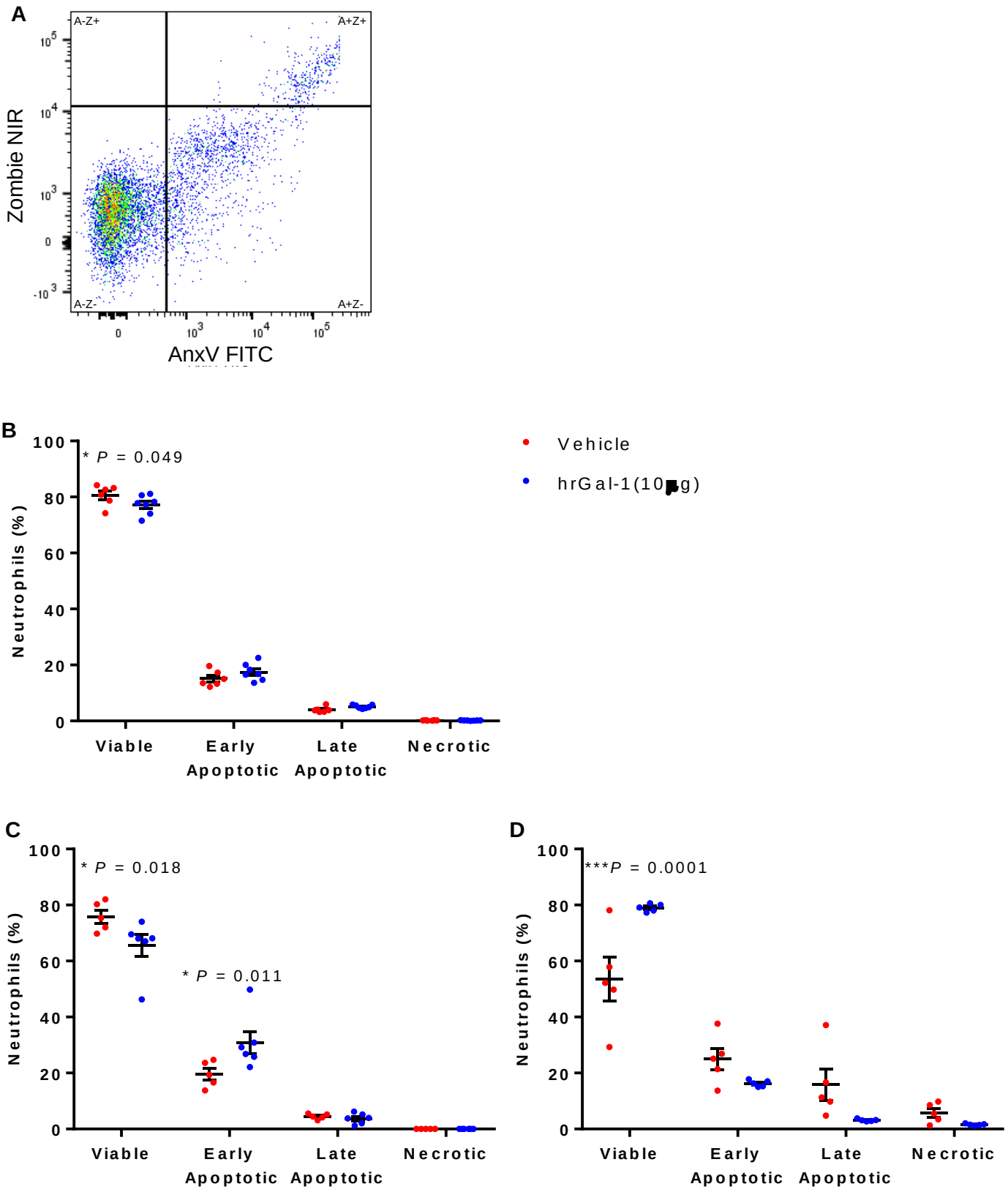

A
A-Z+
A+Z+
A-Z-
A+Z-
Zombie NIR
AnxV FITC
B
* P = 0.049
Neutrophils (%)
Viable
Early Apoptotic
Late Apoptotic
Necrotic
Vehicle
hrGal-1(10µg)
C
* P = 0.018
* P = 0.011
D
***P = 0.0001

**Figure 25. Exogenous Gal-1 accelerated neutrophil apoptosis *in vivo.***
Mice received zymosan (1mg i.p.) and were administered hrGal-1 (10µg) or vehicle (200µl $DPBS^{-/-}$) 8h later during the peak of neutrophil infiltration. Peritoneal lavage was performed at 8h for baseline and subsequently at 24h and 48h. Flow cytometry was used to select the neutrophil (Ly6G+) population, which was further assessed for AnxV binding and Zombie (NIR) staining. Quadrant gating (**A**) was applied to determine viable (AnxV-NIR-), early apoptotic (AnxV+NIR-), late apoptotic (AnxV+NIR+) and necrotic (AnxV-NIR+) neutrophil populations. Results for the percentages of the neutrophil population within each of the quadrants is shown at 6h (**B**) when hrGal-1 was administered during neutrophil recruitment (at 2h) and at 24h (**C**) and 48h (**D**) post when hrGal-1 administration followed the peak in neutrophil number (at 8h). Statistical analysis was performed using an unpaired T-test for each quadrant at each time point with results displayed showing the mean ± SEM, in all cases significant results are considered as $P < 0.05$. n=5-7.

In addition to analysis of neutrophils taken directly from the peritoneal cavity to assess *in vivo* apoptosis cells from peritoneal exudate were also incubated overnight to assess *ex vivo* apoptosis. Analysis of *ex vivo* neutrophil apoptosis was performed at 6h, having received vehicle or hrGal-1 at 2h post injection of zymosan (figure 26A & B).

Neutrophils that were collected from the peritoneal cavity, incubated overnight and then assessed for apoptosis, were in the majority early apoptotic (62.53% ± 6.89%), as shown in figure 26B. A considerable population were late apoptotic (27.24% ± 10.52%) and a modest population of viable cells was also present (10.20% ± 3.65%). A very small percentage of neutrophils were necrotic (0.12% ± 0.08%). Gal-1-treated mice had a significantly lower percentage of viable neutrophils (6.54% ± 0.47%) compared to those from vehicle controls (13.85% ± 2.01%) as well as a significantly lower percentage of early apoptotic cells (55.64% ± 1.47% vs. 69.42% ± 0.89%). Correspondingly, there was a significantly higher percentage of late apoptotic neutrophils in Gal-1 treated mice (37.76% ± 1.67% vs. 16.72% ± 1.49%) suggesting that they had progressed further into the process of apoptosis.

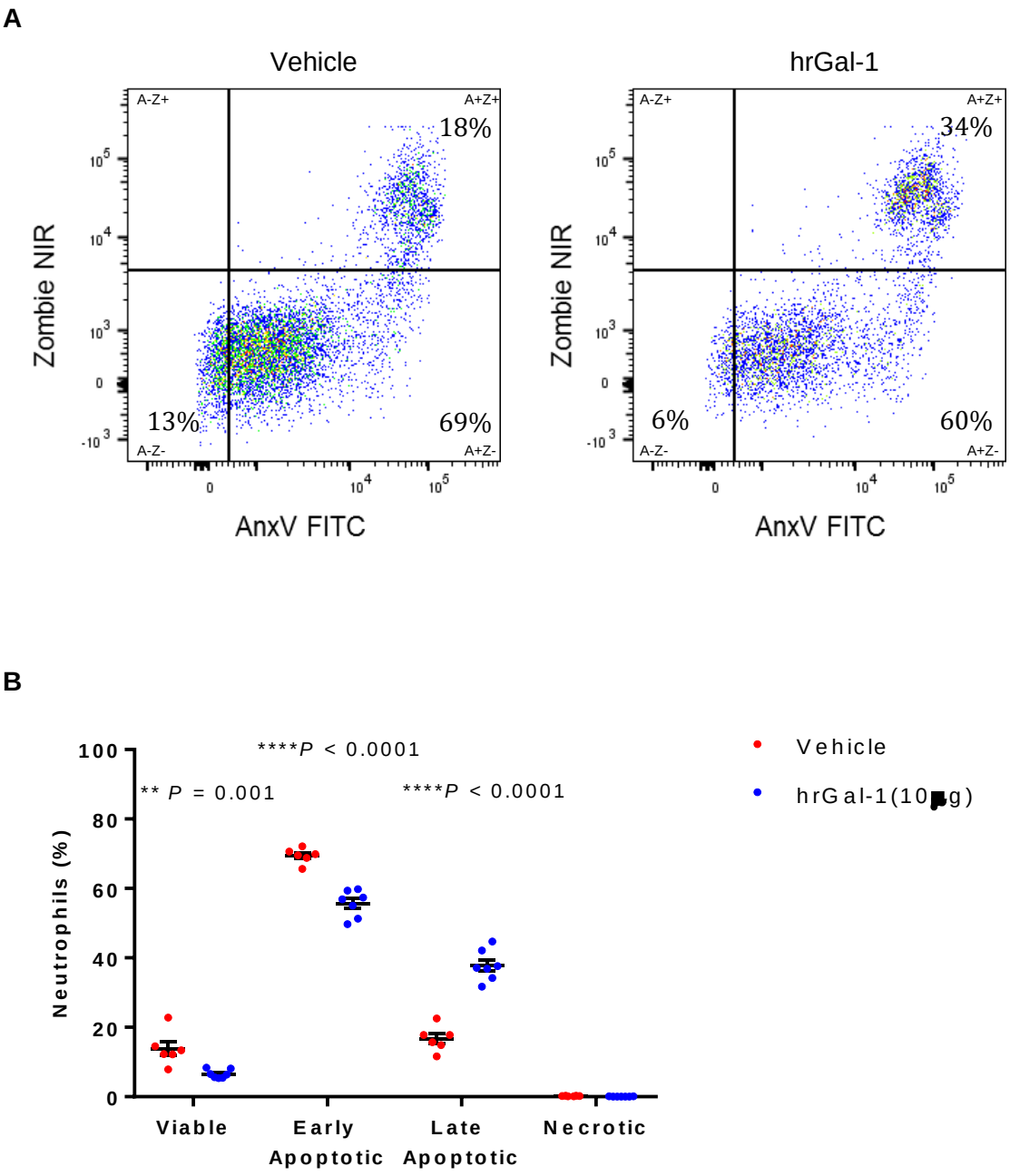



**Figure 26. Exogenous Gal-1 treatment enhanced neutrophil apoptosis *ex vivo.***
Peritonitis was initiated with zymosan (1mg i.p.) and mice were treated with hrGal-1 (10µg) or vehicle (200µl DPBS-/-) at 2h and peritoneal lavage performed at 6h. Peritoneal exudate (1mL) cells were resuspended in RPMI + 0.5% BSA and incubated overnight (20h). Flow cytometry was used to select the neutrophil (Ly6G+) population, which was further assessed for AnxV binding and Zombie (NIR) staining. Quadrant gating (**A**) was applied to determine viable (AnxV-NIR-), early apoptotic (AnxV+NIR-), late apoptotic (AnxV+NIR+) and necrotic (AnxV-NIR+) neutrophil populations. Representative plots are displayed for veh (left) and hrGal-1 (right) treated mice (**A**). Representative plots for each treatment indicating the percentages of the neutrophil populations within each of the quadrants is shown (**B**). Statistical analysis was performed using an unpaired t-test for each quadrant with results displayed showing the mean ± SEM, in all cases results are considered significant when $P < 0.05$. n=6-7.

### *3.2.3. Neutrophil Clearance*

Administration of Gal-1 at either 2h or 8h post-zymosan did not modulate efferocytosis at either the 6h or 24 and 48h timepoints respectively (figure 27).

In addition to analysing peritoneal neutrophils for their levels of apoptosis engulfment of these cells by macrophages was also investigated. Following fixation and permeabilisation of peritoneal cells macrophages containing neutrophils (F4/80+Ly6G+) were selected to assess *in vivo* efferocytosis. Analysis of efferocytosis was performed at 6h (figure 27A & B), having received vehicle or hrGal-1 at 2h post injection of zymosan. Here data showed a subtle trend towards a decrease in the percentage of peritoneal cells involved in the process of efferocytosis following treatment with hrGal-1, however the relative number of neutrophils per macrophage was slightly more in response to treatment with hrGal-1. Fewer macrophage ingesting more neutrophils each suggests that there would be no overall difference in cell clearance at this early timepoint

When efferocytosis was assessed at 24h (figure 27C & D) and 48h (figure 27E & F) post zymosan after treatment with hrGal-1 or vehicle at 8h, the MFI values were considerably higher compared to the 6h timepoint (figure 27B), indicating that there were a greater number of neutrophils contained within macrophages at the latter timepoints (figure 27D & F), with peak numbers seen at the 24h timepoint. However, despite the notable change in relative MFI values across time, there were no differences between genotypes nor were there any differences in percentage of peritoneal cells involved in the efferocytic process at these latter timepoints.

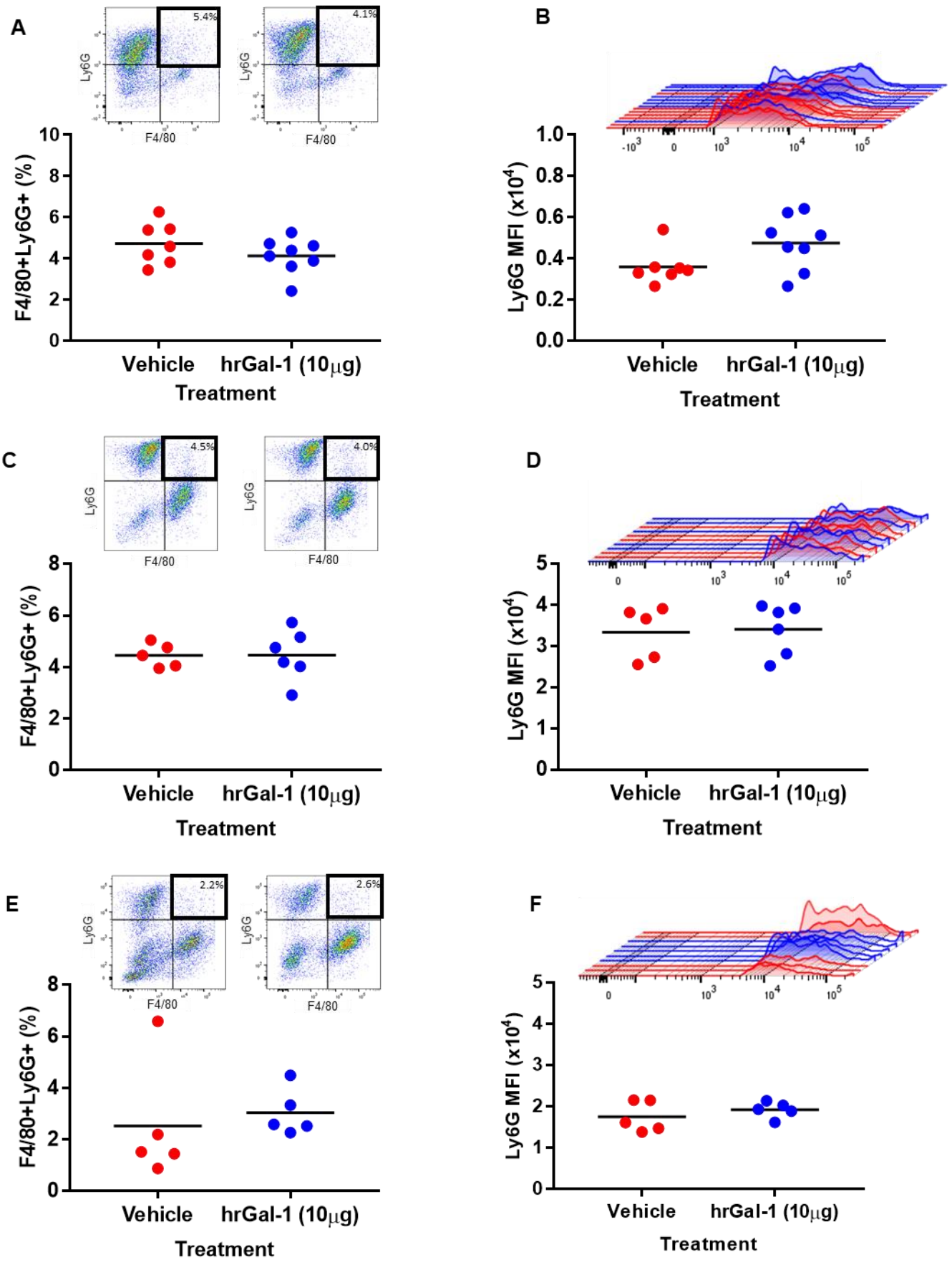

A
5.4%
4.1%
Ly6G
F4/80
F4/80+Ly6G+ (%)
Vehicle
hrGal-1 (10μg)
Treatment
B
Ly6G MFI (x10^4)
C
4.5%
4.0%
D
E
2.2%
2.6%
F

**Figure 27. *In vivo* efferocytosis is not modulated by exogenous Gal-1.**
Mice received zymosan (1mg i.p.) and were administered hrGal-1 (10µg) or vehicle (200µl DPBS-/-) at 2h and peritoneal lavage performed at 6h (hrGal-1 at 2h), 24h and 48h (hrGal-1 at 8h). Cell surface staining for macrophages ($F4/80^+$) was followed by fixation, permeabilisation and neutrophil ($Ly6G^+$) staining. Quadrant gating was applied to determine the percentage of cells positive for efferocytosis ($F4/80^+Ly6G^+$), representative plots inlaid above for both vehicle and hrGal-1 treated mice. Results show the individual and mean values for the percentage of cells positive for efferocytosis at 6h (**A**), 24h (**C**) and 48h (**E**). The median fluorescence intensity (MFI) for neutrophil ($Ly6G^+$) staining of the cells positive for efferocytosis ($F4/80^+Ly6G^+$) at 6h (**B**), 24h (**D**) and 48h (**F**) are also shown with representative MFI value plots above. Statistical analysis was performed using an unpaired t-test at each time point and in all cases results are considered significant when $P < 0.05$. n=5-7.

### *3.2.4. Migration to the Lymph Node.*

Neutrophil ($Ly6G^+$) numbers and lymphocyte ($CD3^+CD4^+$ and $CD3^+CD8^+$) subtypes within the inguinal lymph node were investigated at 24 and 48h post zymosan following treatment with hrGal-1 (or vehicle) at 8h. No changes in the percentages of neutrophils (figure 28A & B), helper (CD4+) T cells (figures 28C & D) and cytotoxic ($CD8^+$) T cells were observed following treatment with exogenous Gal-1 (figure 28E & F).

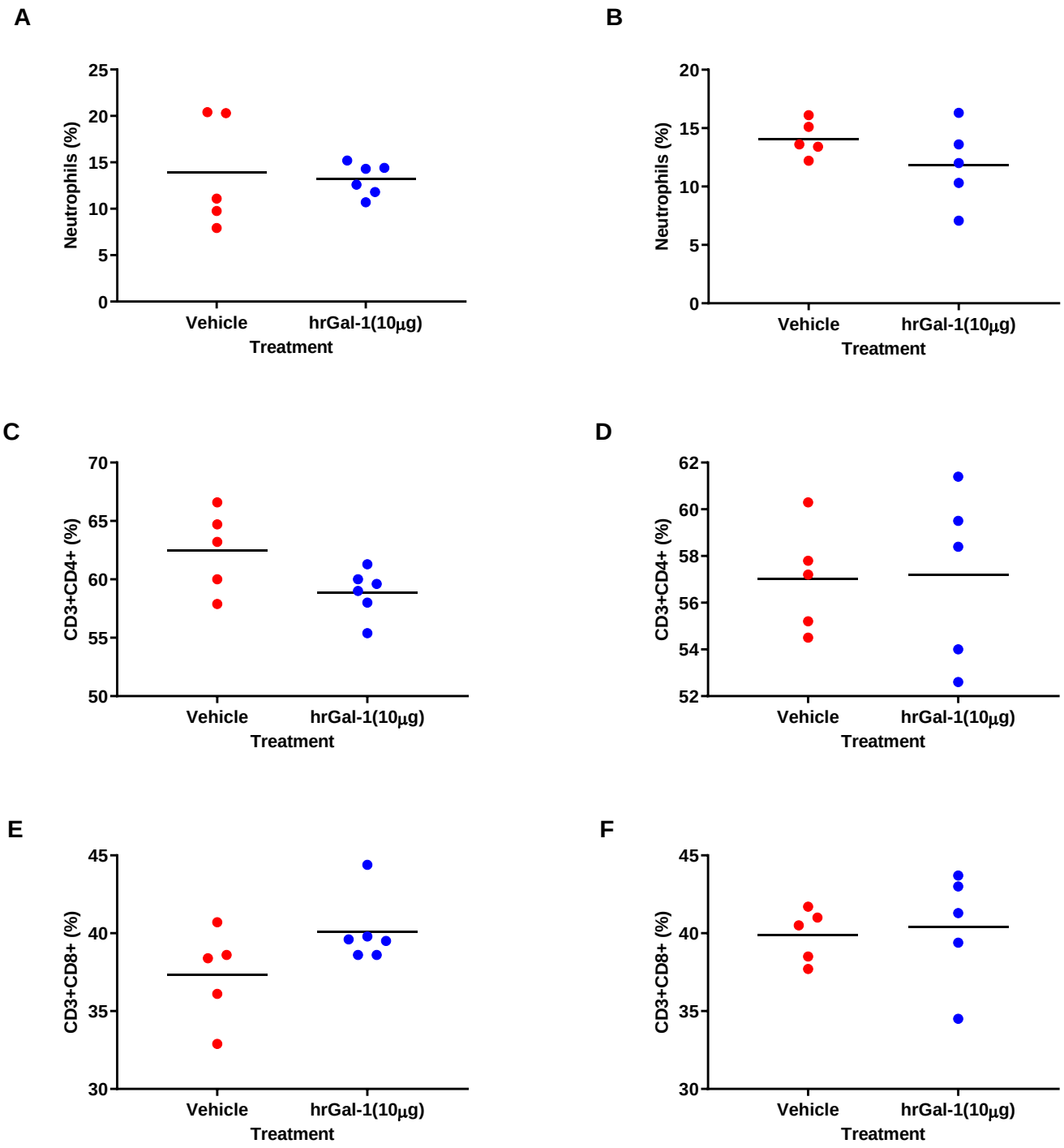



**Figure 28. Exogenous Gal-1 had no effect on the percentage of neutrophils and lymphocytes within the lymph node.**

Mice received zymosan (1mg i.p.) and were administered hrGal-1 (10µg) or vehicle (200µl DPBS-/-) 8h later during the peak of neutrophil infiltration. Inguinal, axillary, mesenteric and popliteal lymph nodes were collected at 24h and 48h. Cells were isolated using a cell strainer and flow cytometry was used to analyse the cell populations. Positive populations were determined using antibodies specific to neutrophils ($Ly6G^+$) and cytotoxic ($CD8^+$) and helper ($CD4^+$) subsets of T cells ($CD3^+$). Results show percentages of neutrophils (**A**,**B**), helper T cells (**C**,**D**) and cytotoxic T cells (**E**,**F**) at 24h and 48h. Statistical analysis was performed using an unpaired t-test for each time point with results displayed additionally showing the mean of each treatment group, in all cases significant results are considered as $P < 0.05$. n=5-6.

### *3.3. The Effect of Exogenous Gal-1 in a Model of Delayed Resolving Inflammation.*

To determine whether Gal-1 influences neutrophil trafficking, clearance or both in a high dose zymosan model (10mg) where resolution of the inflammatory response is comparatively delayed compared to the acute disease profile with 1mg zymosan described until now, hrGal-1 was administered daily from peak neutrophil influx at 24h post-zymosan and peritoneal cavities lavaged at 72 and 120h.

#### *3.3.1. Leukocyte Trafficking*

Results from administering hrGal-1 treatment daily from 24h post high dose zymosan showed no significant differences in leukocyte recruitment to the peritoneal cavity of any leukocyte subtype at either 72h or 120h (figure 29A-G). The data shows a notable trend towards fewer macrophages (most prominent for the mature ($F4/80^{+}CD11b^{high}$; figure 29F population) in response to hrGal-1, however this was not statistically significant.

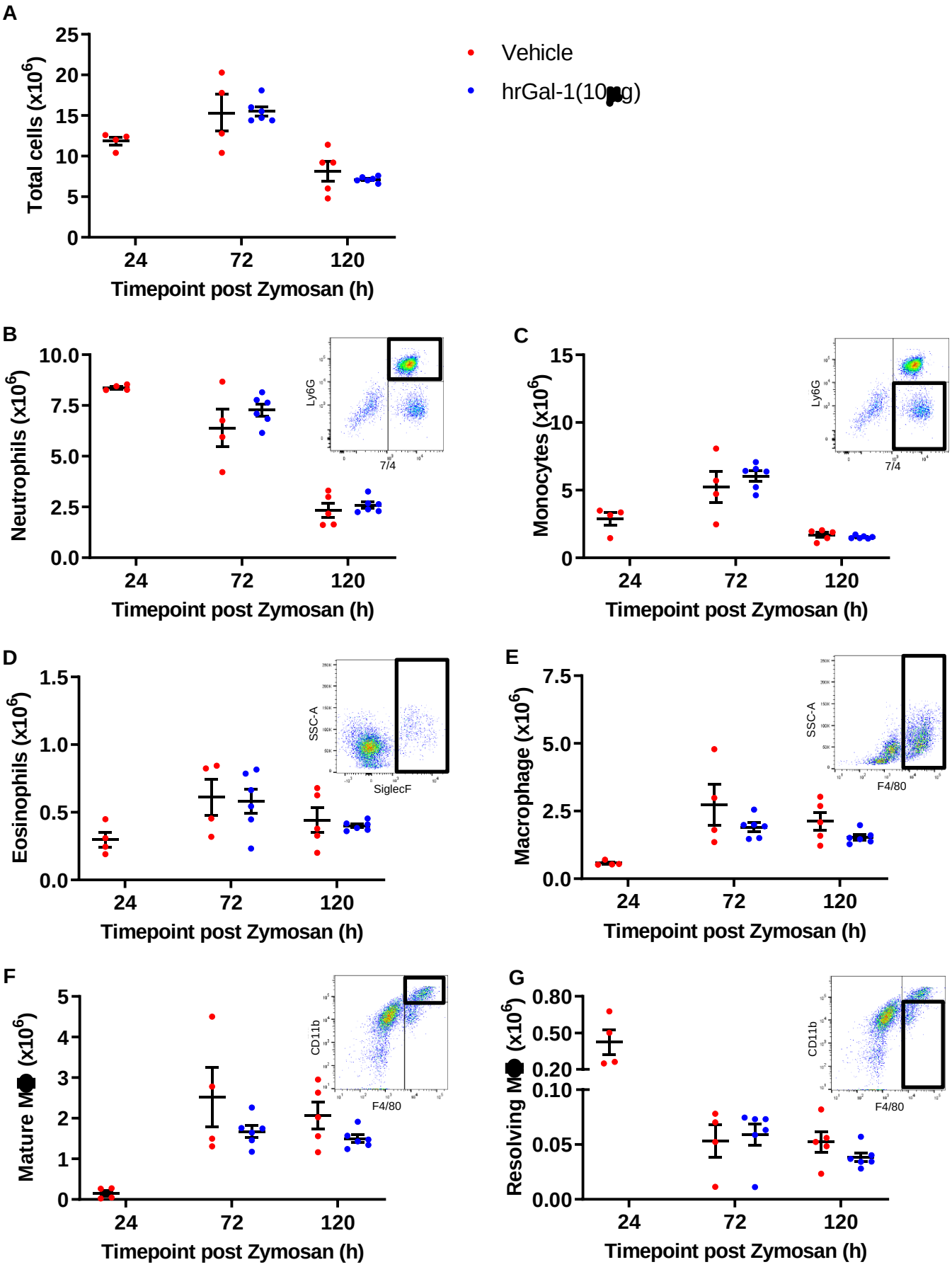

A
Total cells (x10^6)
25
20
15
10
5
0
24
72
120
Timepoint post Zymosan (h)
Vehicle
hrGal-1(10 g)
B
Neutrophils (x10^6)
10.0
7.5
5.0
2.5
0.0
Ly6G
7/4
C
Monocytes (x10^6)
15
10
5
0
D
Eosinophils (x10^6)
1.5
1.0
0.5
0.0
SSC-A
SiglecF
E
Macrophage (x10^6)
7.5
5.0
2.5
0.0
F4/80
F
Mature M (x10^6)
5
4
3
2
1
0
CD11b
F4/80
G
Resolving M (x10^6)
0.80
0.50
0.20
0.10
0.05
0.00

**Figure 29. Exogenous Gal-1 had no effect on leukocyte numbers in a high-dose zymosan peritonitis model.** Mice received zymosan (10mg i.p.) and were administered hrGal-1 (10µg) or vehicle (200µl $DPBS^{-/-}$) daily from 24h (peak of neutrophil infiltration). Peritoneal lavage was performed at 24h for baseline and subsequently at 72h and 120h. Cells from the exudate were counted to enumerate total cells ($CD45^+$) (**A**) and neutrophils ($7/4^+Ly6G^+$) (**B**), inflammatory monocytes ($7/4^+Ly6G^-$) (**C**), eosinophils (Siglec $F^+$) (**D**), macrophages ($F4/80^+$) (**E**), mature macrophages ($F4/80^+CD11b^{high}$) (**F**) and resolving macrophages ($F4/80^+CD11b^{low}$) (**G**) were determined using flow cytometry. Representative flow cytometry gating plots are inset for each subset. Statistical analysis was performed using a two-way ANOVA with Sidak's multiple comparisons test, results display the mean ± SEM, in all cases significant results are considered as $P < 0.05$. n=4-6.

### *3.3.2. Neutrophil Apoptosis*

Analysis of *in vivo* neutrophil apoptosis was performed at 24h (for comparison), as well as at 72 and 120h (with hrGal-1 given daily from 24h) post injection of high dose zymosan. At the 24h time point (figure 30A), neutrophils were predominantly viable (83.75% ± 2.06%) with smaller proportions early apoptotic (10.42% ± 1.41%), late apoptotic (5.35% ± 0.64%) or necrotic (0.47% ± 0.23%). Neutrophils obtained at subsequent time points were also primarily viable, with the highest percentage viability seen at 72h (90.71% ± 0.75). Treatment with hrGal-1 did not modulate levels of apoptosis seen at this time-point (figure 30B). At 120h post zymosan (figure 30C) there was a significantly higher percentage of viable cells within the population of neutrophils retrieved from the cavities of mice treated with hrGal-1 compared to vehicle (88.47% ± 0.98% vs 84.32% ± 1.43%; $p = 0.0362$). Correspondingly fewer early apoptotic cells were present within the cavities of hrGal-1 treated mice (3.80% ± 0.35% vs 5.73% ± 0.51%; $p = 0.0108$).

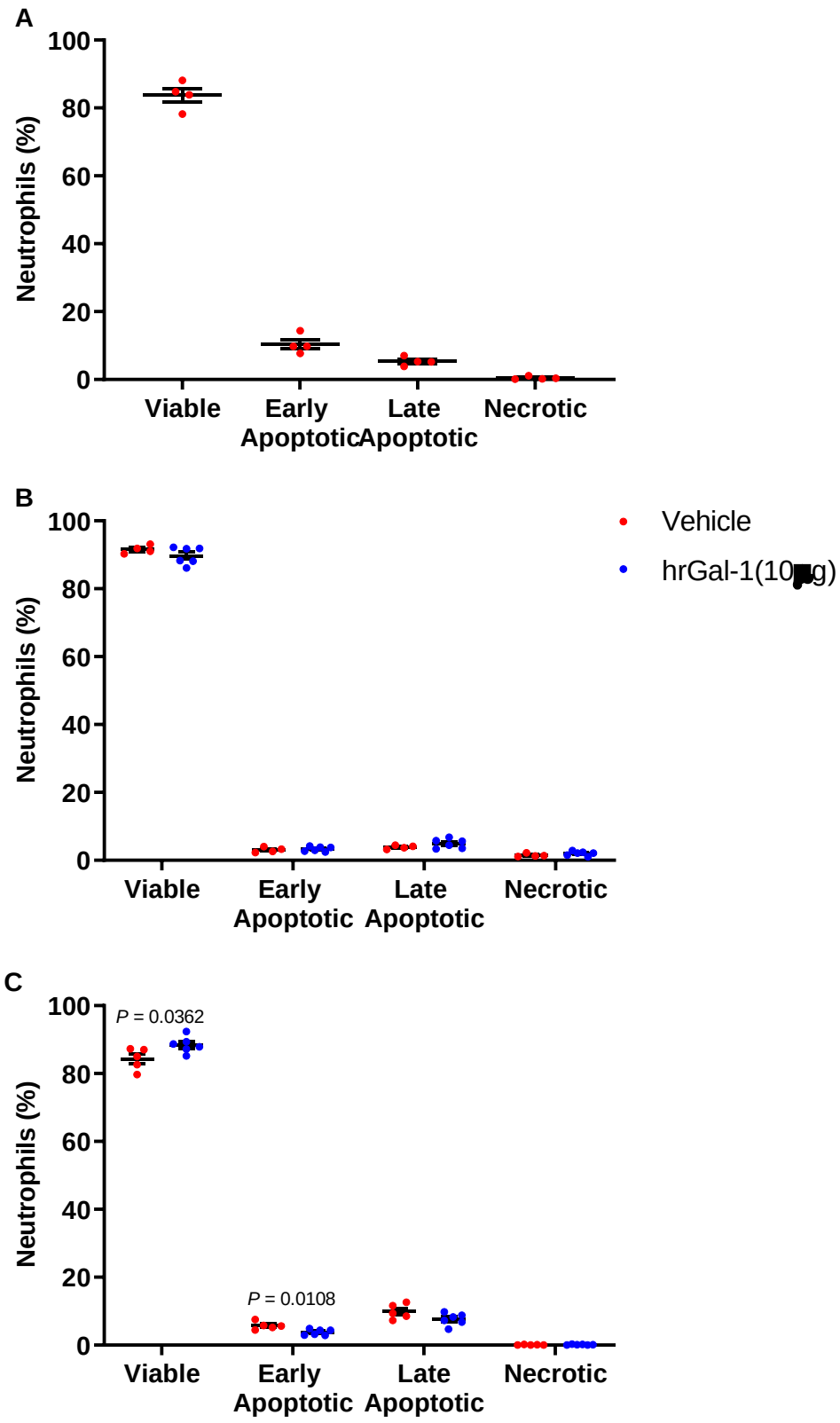

A
Neutrophils (%)
100
80
60
40
20
0
Viable
Early Apoptotic
Late Apoptotic
Necrotic
B
Vehicle
hrGal-1(10
g)
C
P = 0.0362
P = 0.0108

**Figure 30. Exogenous Gal-1 treatment in the high-dose zymosan peritonitis model resulted in fewer apoptotic neutrophils during the late phase of the inflammatory response.**
Mice received zymosan (10mg i.p.) and were administered hrGal-1 (10µg) or vehicle (200µl $DPBS^{-/-}$) daily from 24h (peak of neutrophil infiltration). Peritoneal lavage was performed at 24h for baseline and subsequently at 72h and 120h. Flow cytometry was used to select the neutrophil (Ly6G+) population, which was further assessed by AnxV binding and Propidium Iodide (PI) staining. Quadrant gating was applied to determine viable (AnxV-PI-), early apoptotic (AnxV+PI-), late apoptotic (AnxV+PI+) and necrotic (AnxV-PI+) neutrophil populations. Results for the percentages of the neutrophil population within each of the quadrants is shown at 24h (**A**), 72h (**B**) and 120h (**C**). Statistical analysis was performed using an unpaired t-test for each quadrant at each time point with results displayed showing the mean ± SEM, in all cases significant results are considered as $P < 0.05$. n=4-6.

### *3.3.3. Neutrophil Clearance*

The daily administration of hrGal-1 following high dose zymosan did not modulate efferocytosis when detected at 72 or 120h post inflammogen (figure 31A-F).

Following fixation and permeabilisation of peritoneal cells, macrophages containing neutrophils (F4/80+Ly6G+) were selected to assess *in vivo* efferocytosis. When assessed at 72h (figure 31C & D) post high dose zymosan, with either hrGal-1 or vehicle administered daily from 24h onwards, the percentage of double positive (F4/80+Ly6G+) cells was notably higher compared to both the earlier 24h timepoint (figure 31A & B) and the latter 120h timepoint (figure 31E & F). This data indicates that there was a greater percentage of macrophages having engulfed neutrophils at this time point, whereas similar lower percentages were detected at 24h and 120h.

Despite the notable change across time, there were no differences detected in response to treatment with hrGal-1. Interestingly, the peritoneal macrophage population was consistent for their Ly6G MFI levels across all assessed timepoints regardless of whether they had received treatment with hrGal-1 or not. This would suggest that efferocytosis was a constant process within the peritoneal cavity, and that in an attempt to counteract the increase in neutrophil numbers, seen as a result of the recurrent recruitment of cells to the cavity, there was ongoing clearance of these cells.

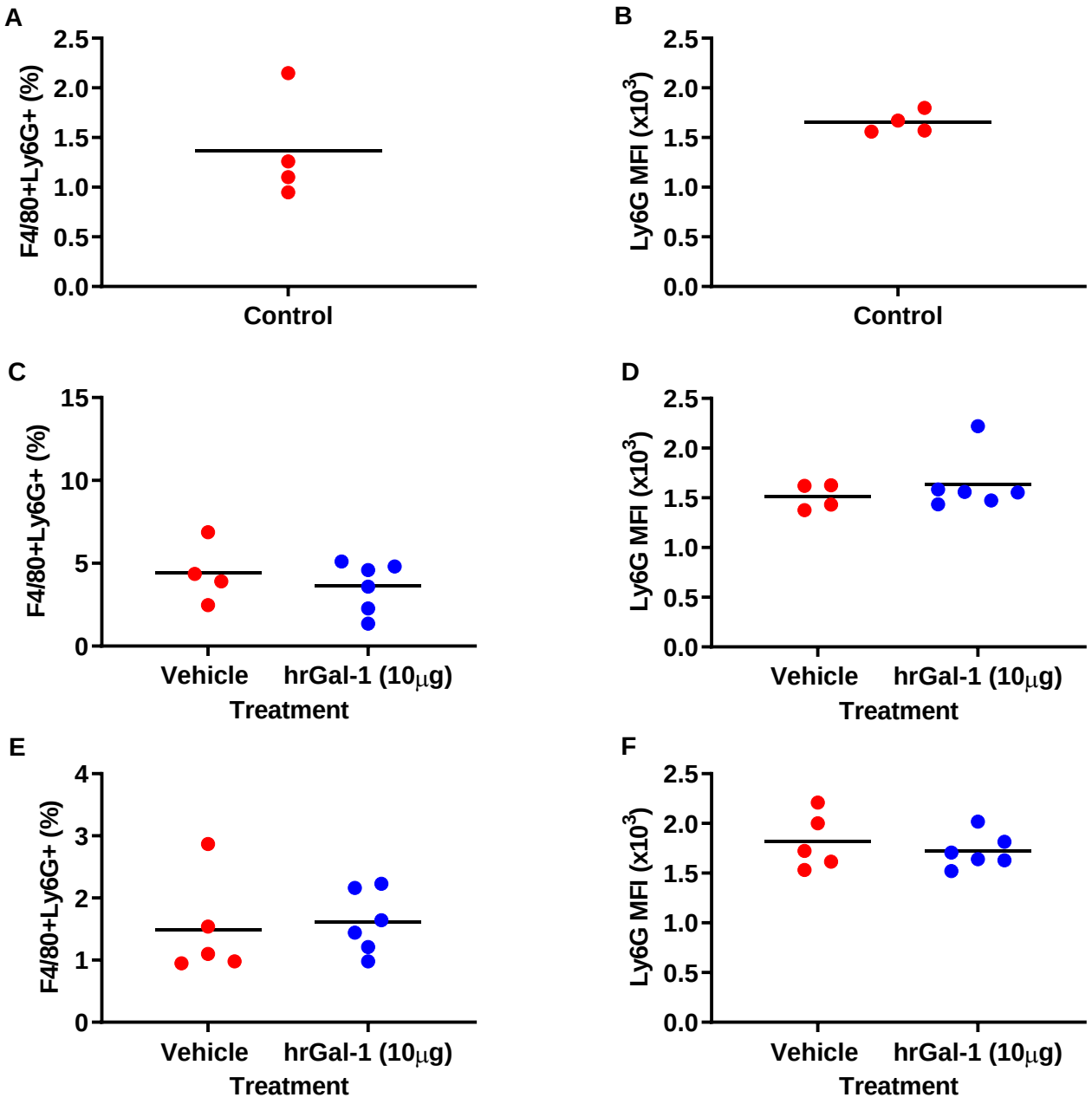



**Figure 31. Exogenous Gal-1 did not modulate efferocytosis in the high dose zymosan peritonitis model.** Mice received zymosan (10mg i.p.) and were administered hrGal-1 (10μg) or vehicle (200μl DPBS$^{-/-}$) daily from 24h (peak of neutrophil infiltration). Peritoneal lavage was performed at 24h for baseline and subsequently at 72h and 120h. Cell surface staining for macrophage (F4/80+) was followed by fixation, permeabilisation and neutrophil (Ly6G+) staining. Quadrant gating was applied to determine the percentage of cells positive for efferocytosis (F4/80+Ly6G+). Results show the individual and mean values for the percentage of cells positive for efferocytosis at 24h (**A**), 72h (**C**) and 120h (**E**). The mean fluorescence intensity (MFI) for neutrophil (Ly6G+) staining of the double positive (F4/80+Ly6G+) cells at 24h (**B**), 72h (**D**) and 120h (**F**) are also shown. Statistical analysis was performed using an unpaired t-test and in all cases results are considered significant when $P < 0.05$. n=4-6.

### *3.3.4. Peripheral blood Leukocyte Profile*

The peripheral blood was collected from mice at 72h and 120h post high dose zymosan (10mg) and profiled for 27 parameters (see table 6). There were no significant differences detected at either time point in mice administered hrGal-1 (10µg) compared to vehicle only.

However, the data from 72h displays a recognisable bias towards an increased myeloid cell population in the peripheral blood of hrGal-1 treated mice, resulting in an overall increase in white blood cell (WBC) counts (figure 32A). As shown in figure 32B platelet counts were also increased in response to hrGal-1 treatment. In line with the overall increase in WBC counts populations of myeloid cells including neutrophils, monocytes and eosinophils were increased in both number and percentage as shown in figures 32C-H, Conversely, a decreased population of lymphoid cells (figures 32I & J) was seen the peripheral blood of hrGal-1 treated mice.

Interestingly, data from 120h shows the inverse, the WBC count, as shown in figure 33A, was lower in mice having received hrGal-1. However, there was no effect on platelet counts at 120h (figure 33B). In support of the decreased WBCs, lower numbers of all myeloid subsets (figures 33C-H) as well as a subtle decrease in lymphoid cell number (figures 33I & J) were detected in the peripheral blood of hrGal-1 treated mice.

No alterations in red blood cell counts or parameters were seen in response to hrGal-1 at either 72h or 120h post zymosan (data not shown).

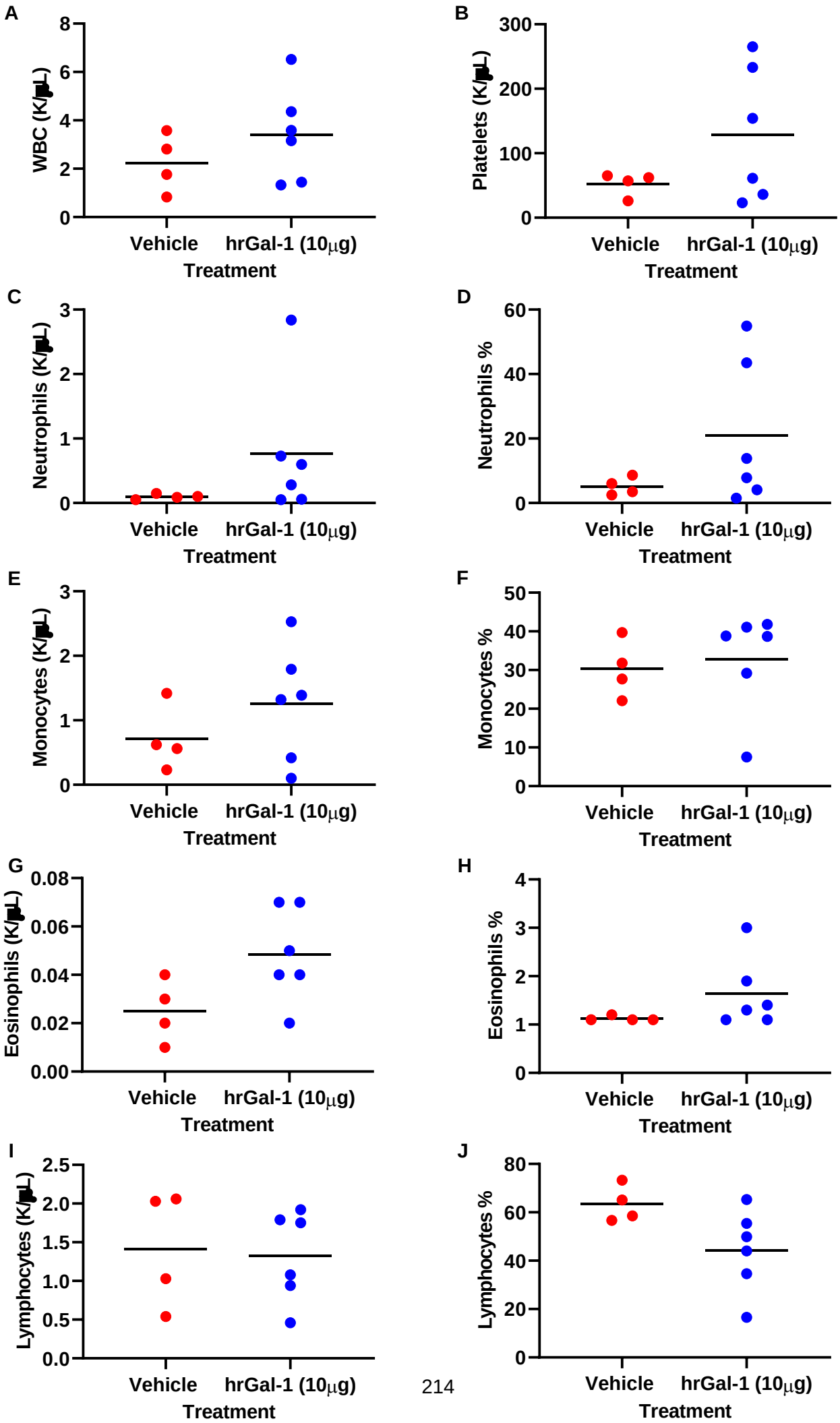

A
WBC (K/μL)
8
6
4
2
0
Vehicle
hrGal-1 (10μg)
Treatment
B
Platelets (K/μL)
300
200
100
0
Vehicle
hrGal-1 (10μg)
Treatment
C
Neutrophils (K/μL)
3
2
1
0
Vehicle
hrGal-1 (10μg)
Treatment
D
Neutrophils %
60
40
20
0
Vehicle
hrGal-1 (10μg)
Treatment
E
Monocytes (K/μL)
3
2
1
0
Vehicle
hrGal-1 (10μg)
Treatment
F
Monocytes %
50
40
30
20
10
0
Vehicle
hrGal-1 (10μg)
Treatment
G
Eosinophils (K/μL)
0.08
0.06
0.04
0.02
0.00
Vehicle
hrGal-1 (10μg)
Treatment
H
Eosinophils %
4
3
2
1
0
Vehicle
hrGal-1 (10μg)
Treatment
I
Lymphocytes (K/μL)
2.5
2.0
1.5
1.0
0.5
0.0
Vehicle
hrGal-1 (10μg)
Treatment
J
Lymphocytes %
80
60
40
20
0
Vehicle
hrGal-1 (10μg)
Treatment

**Figure 32. Exogenous Gal-1 increased peripheral white blood cell and platelet counts during the early resolution phase of the high dose zymosan peritonitis model.**
Mice received zymosan (10mg i.p.) and were administered hrGal-1 (10µg) or vehicle (200µl $DPBS^{-/-}$) daily from 24h (peak of neutrophil infiltration). Blood was collected at 72h post zymosan injection. Whole blood (50µl) was ran on the ProCyte DX Haematolgy Analyser (IDEXX) and analysed for while blood cell (WBC) (**A**) and platelets (**B**). As well as neutrophil (**C**, **D**), monocyte (**E**, **F**), eosinophils (**G**, **H**) and lymphocyte (**I**, **J**) numbers and percentage respectively. Statistical analysis was performed using an unpaired t-test and results considered as significant when $P < 0.05$. n=4-6.

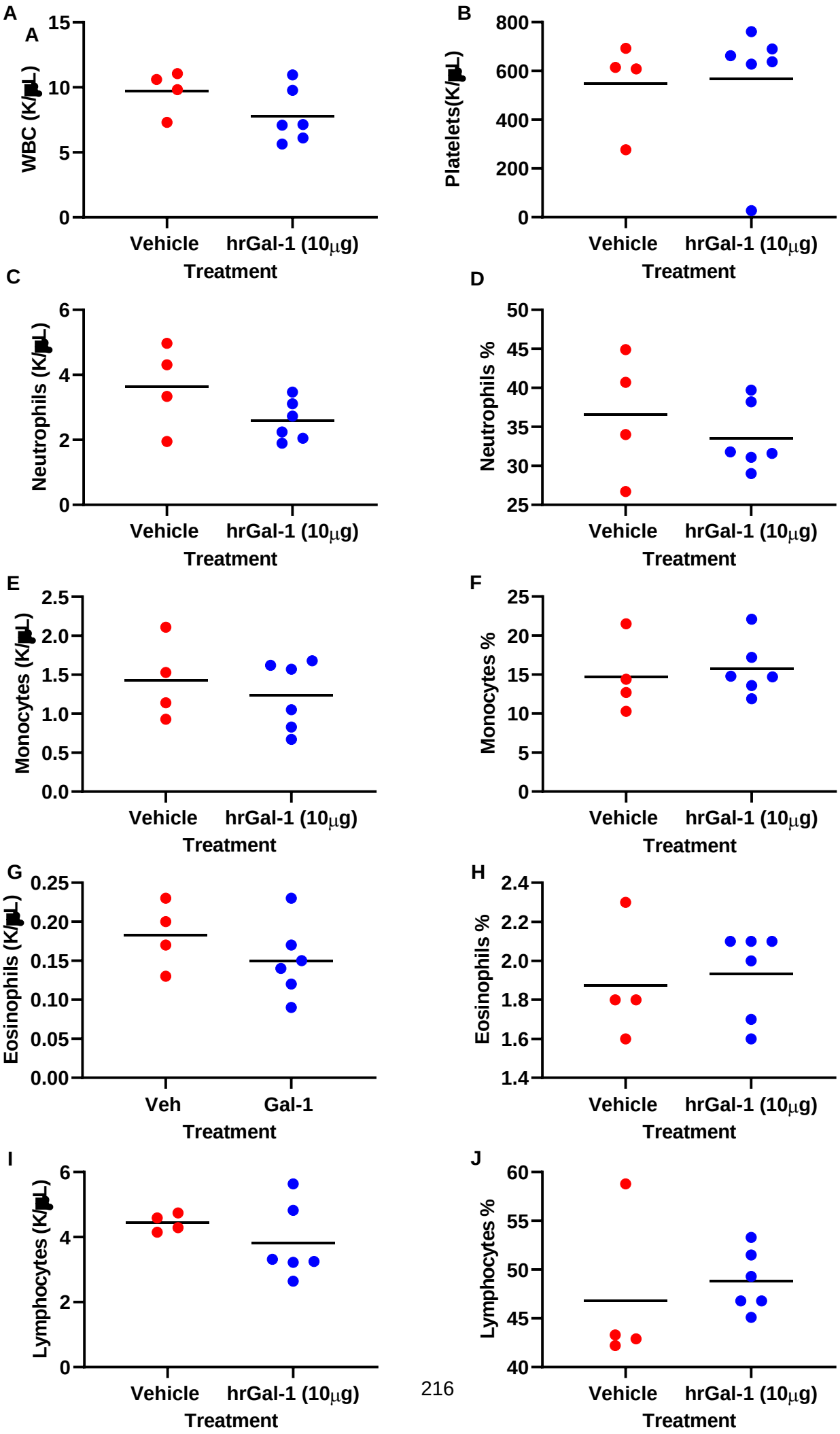

A
A
WBC (K/μL)
15
10
5
0
Vehicle
hrGal-1 (10μg)
Treatment
B
Platelets(K/μL)
800
600
400
200
0
Vehicle
hrGal-1 (10μg)
Treatment
C
Neutrophils (K/μL)
6
4
2
0
Vehicle
hrGal-1 (10μg)
Treatment
D
Neutrophils %
50
45
40
35
30
25
Vehicle
hrGal-1 (10μg)
Treatment
E
Monocytes (K/μL)
2.5
2.0
1.5
1.0
0.5
0.0
Vehicle
hrGal-1 (10μg)
Treatment
F
Monocytes %
25
20
15
10
5
0
Vehicle
hrGal-1 (10μg)
Treatment
G
Eosinophils (K/μL)
0.25
0.20
0.15
0.10
0.05
0.00
Veh
Gal-1
Treatment
H
Eosinophils %
2.4
2.2
2.0
1.8
1.6
1.4
Vehicle
hrGal-1 (10μg)
Treatment
I
Lymphocytes (K/μL)
6
4
2
0
Vehicle
hrGal-1 (10μg)
Treatment
J
Lymphocytes %
60
55
50
45
40
Vehicle
hrGal-1 (10μg)
Treatment

**Figure 33. Exogenous Gal-1 resulted in a trend towards decreased peripheral white blood cell counts during the resolution phase of the high dose zymosan peritonitis model.**
Mice received zymosan (10mg i.p.) and were administered hrGal-1 (10µg) or vehicle (200µl $DPBS^{-/-}$) daily from 24h (peak of neutrophil infiltration). Blood was collected at 120h post zymosan injection. Whole blood (50µl) was ran on the ProCyte DX Haematolgy Analyser (IDEXX) and analysed for while blood cell (WBC) (**A**) and platelets (**B**). As well as neutrophil (**C**, **D**), monocyte (**E**, **F**), eosinophils (**G**, **H**) and lymphocyte (**I**, **J**) numbers and percentage respectively. Statistical analysis was performed using an unpaired t-test and results considered as significant when $P < 0.05$. n=4-6.

### *3.4. The Role of Endogenous Gal-1 in Inflammatory Arthritis.*

The role of endogenous Gal-1 was next explored in the K/BxN model of inflammatory arthritis by comparing WT and Gal-1 KO mice. Mice were injected with arthritogenic K/BxN serum on days 0 and 2 and monitored daily for clinical signs of arthritis and weight loss as well as paw swelling by water displacement plethysmometry.

#### *3.4.1. Oedema Formation*

The immediate vasoactive response was measured as a change in paw thickness using calipers prior to and thirty minutes post injection with K/BxN serum (day 0). This initial response was significantly heightened in Gal-1 KO mice (0.28mm ± 0.01mm vs 0.18mm ± 0.03mm; P = 0.0095) (figure 34).

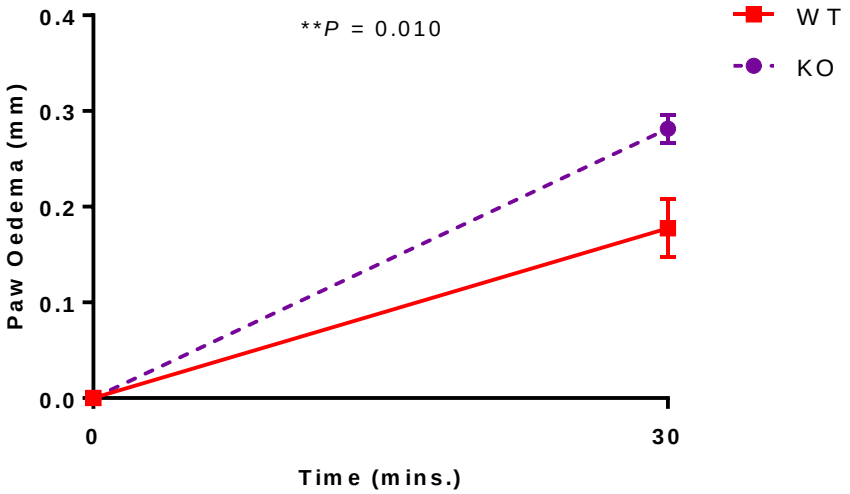



**Figure 34. Initial oedema response is heightened in Gal-1 KO mice.**
WT and Gal-1 KO mice received arthritogenic K/BxN serum (by i.p. injection) and paw swelling was measured using callipers. Fast response paw oedema was assessed 30 mins following serum. Statistical analysis was performed using a Two-way RM ANOVA with results displayed showing the mean ± SEM, in all cases significant results are considered as $P < 0.05$. n=7.

### *3.4.2. Disease Severity*

Administration of K/BxN serum triggered a rapid and robust onset of arthritis. The initiation phase (first 4 days) was less severe in Gal-1 KO mice compared to their WT counterparts. At day three there was significant divergence in scores between genotypes (P = 0.0018), which was maintained at day four when Gal-1 KO (6.41± 0.34) scored significantly less than WT (8.67± 0.64) mice (P = 0.0003). Figure 35A displays the clinical scores for the mice and the overall difference between genotypes (P = 0.0087).

Hind paw oedema was significantly increased in WT mice compared to Gal-1 KO mice and reached significance at day 3 (figure 35B). Overall, across the four-day duration, hind paw oedema is significantly lower in Gal-1 KO mice compared to WT mice (P = 0.0189).

WT mice lost significantly more weight compared to Gal-1 KO mice (P = 0.0042; figure 35C). Gal-1 KO mice also took longer to succumb to disease than WT mice with 100% incidence reached at day 2 in WT mice and day 3 in Gal-1 KO animals (figure 35D).

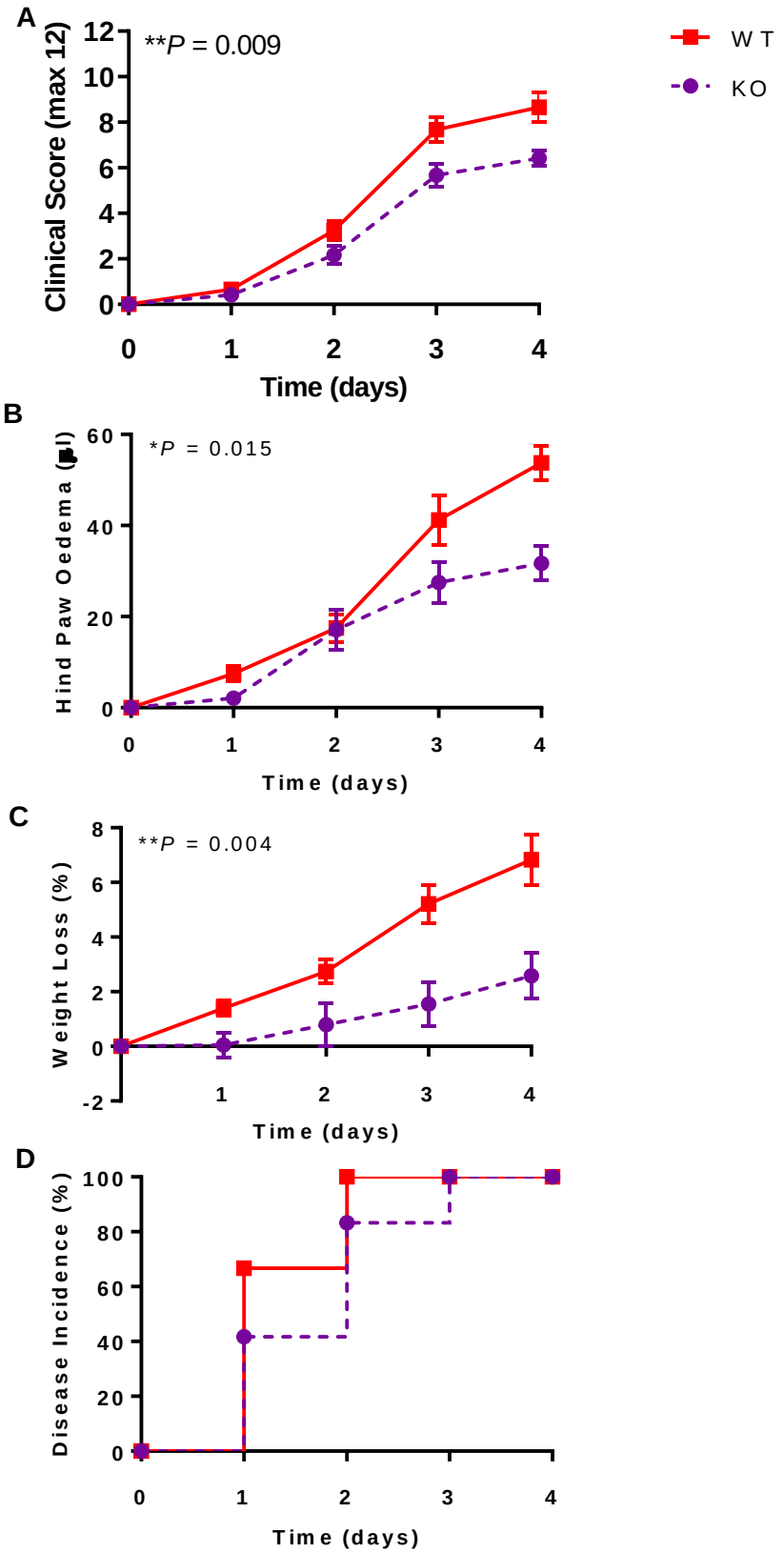



**Figure 35. Early arthritis is less severe in Gal-1 KO mice.**
Arthritis was induced in WT and Gal-1 KO mice by injection of arthritogenic (K/BxN) serum (i.p.) on days 0 and 2. Disease severity was clinically assessed for 4 days post-induction by clinical scoring (**A**), paw oedema by water displacement plethysmometry (**B**) and weight loss (**C**). Incidence of disease (**D**) was also recorded. Statistical analysis was performed using a Two-way RM ANOVA with results displayed showing the mean ± SEM, in all cases significant results are considered as $P < 0.05$. n=7.

### *3.4.3. Microscopic Injury*

Microscopic assessment of joint histology indicated significantly more inflammation in WT mice compared to Gal-1 KO displaying increased leukocyte infiltration and synovial thickness (figure 36A-C).

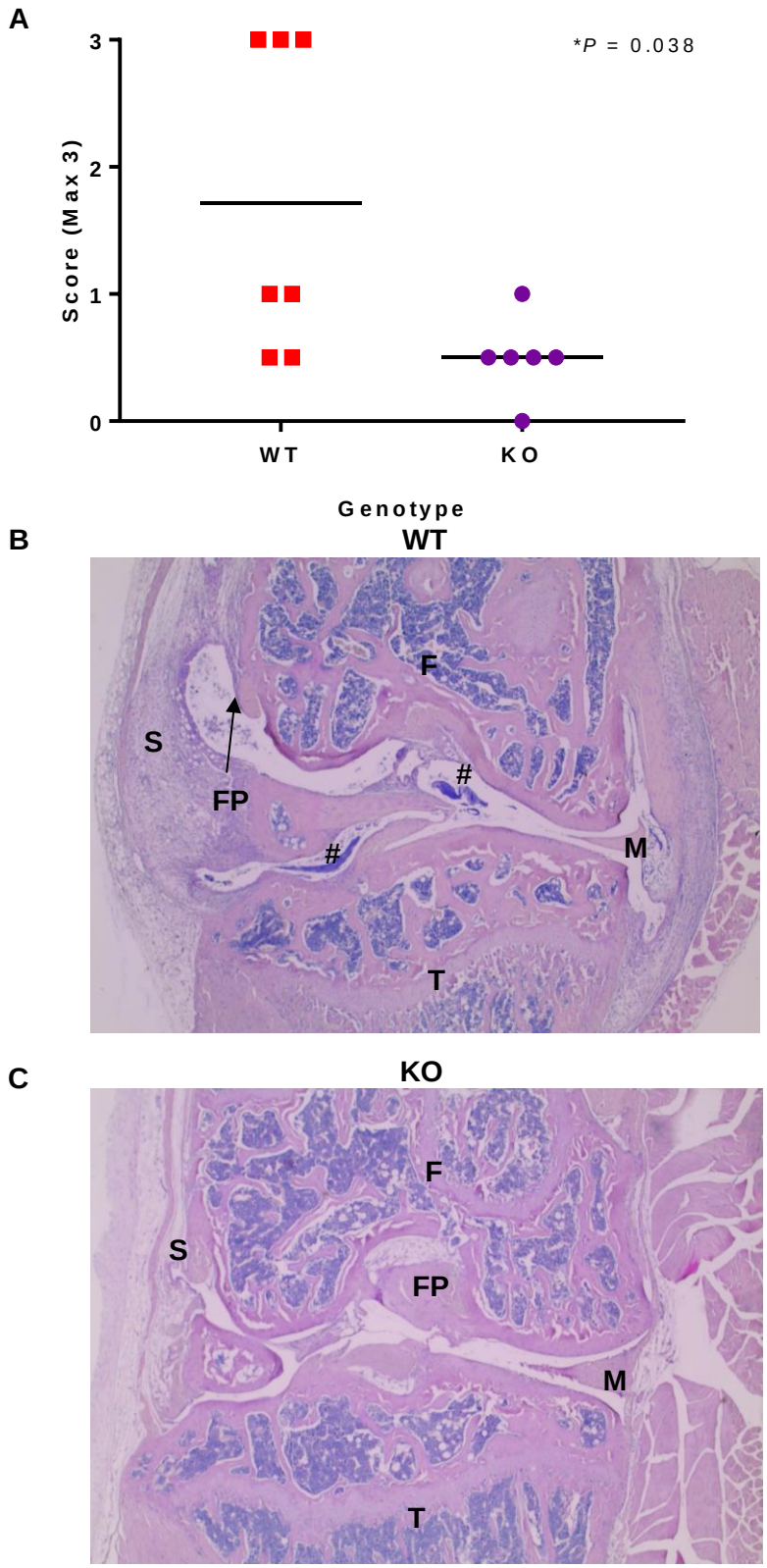



**Figure 36. Cellular infiltrate during the initiation phase of arthritis is less severe in Gal-1 KO mice.**
Arthritis was induced in WT and Gal-1 KO mice by injection of arthritogenic (K/BxN) serum (i.p.) on days 0 and 2, disease progressed for four days and knee joints were collected for histology. H&E stained sections were analysed and scored blindly. Stained sections were scored on a scale of zero to three by assessing level of infiltrate and thickness of the synovium (**A**). Representative images for WT (**B**) and KO (**C**) show scores of 3 and 0 respectively. Statistical analysis was performed using an unpaired t-test with results showing the mean for each genotype, significant results are considered as $P < 0.05$. n=6-7.
# = leukocyte infiltrate, F = femur, FP = fat pad, M = meniscus, S = synovium, T = tibia.

### *3.4.4. Neutrophil Infiltration*

In addition to histological scoring, arthritic paws were also enzymatically digested to further analyse the cellular infiltrate by flow cytometry. At day 4, leukocyte infiltration was assessed within the arthritic paws, with around 10% of CD45 positive cells identified as neutrophils (Ly6G positive). There was no significant difference in the percentage of neutrophils found to have infiltrated the paws of WT mice compared to Gal-1 KO as assessed by flow cytometry (figure 37).

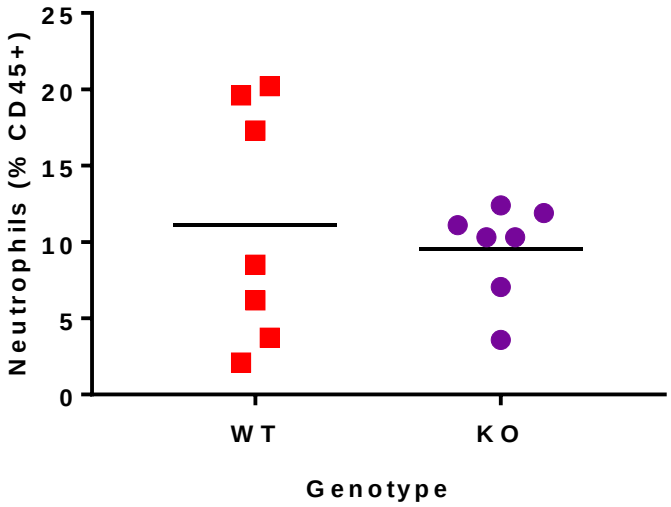



**Figure 37. Neutrophil infiltrate into the paws during early arthritis was not affected by the absence of Gal-1.** Arthritis was induced in WT and Gal-1 KO mice by injection of arthritogenic (K/BxN) serum (i.p) on days 0 and 2, disease progressed for four days and hind paws were collected and tissue digested. Cellular infiltrate was assessed by flow cytometry and percentage of neutrophils ($CD11b^{high}$ $Ly6G^{+}$) was calculated from the $CD45^{+}$ cells. Statistical analysis was performed using an unpaired t-test with results showing the mean for each genotype. Results considered significant if $P < 0.05$. n=6-7.

### *3.4.5. Profile of Draining Lymph Nodes*

Cells isolated from the draining (popliteal) lymph nodes of Gal-1 KO and WT mice were assessed for relative percentages of T cell subsets. Cytotoxic ($CD8^+$) T cells were significantly higher in the draining lymph nodes of WT (40.43% ± 0.82%) mice compared to Gal-1 KO (27.00% ± 1.70%) as shown in figure 38A (P = 0.0021). However, figure 38B indicates that there was no difference between genotypes with regards to helper ($CD4^+$) T cells (WT = 50.73% ± 0.91% and Gal-1 KO = 53.6% ± 3.85%). The $CD4^+$ population was further characterised, and significant differences were seen in the number of naïve ($CD62L^+CD44^-$) and effector ($CD62L^-CD44^-$) T cells, with representative flow cytometry plots for both WT and Gal-1 KO mice (figures 38C & D). The percentage of naïve T cells was significantly lower in Gal-1 KO compared to WT mice (13.91% ± 5.83% vs 44.03% ± 6.75%; P = 0.0279; figure 38E). In contrast, the percentage of effector T cells was significantly enhanced in Gal-1 KO compared to WT (77.37% ± 3.67% vs 47.17% ± 5.41%; P = 0.0099, figure 38G) mice. No differences were observed between genotypes in the percentage of memory ($CD62L^-CD44^+$) T cells (figure 38F).

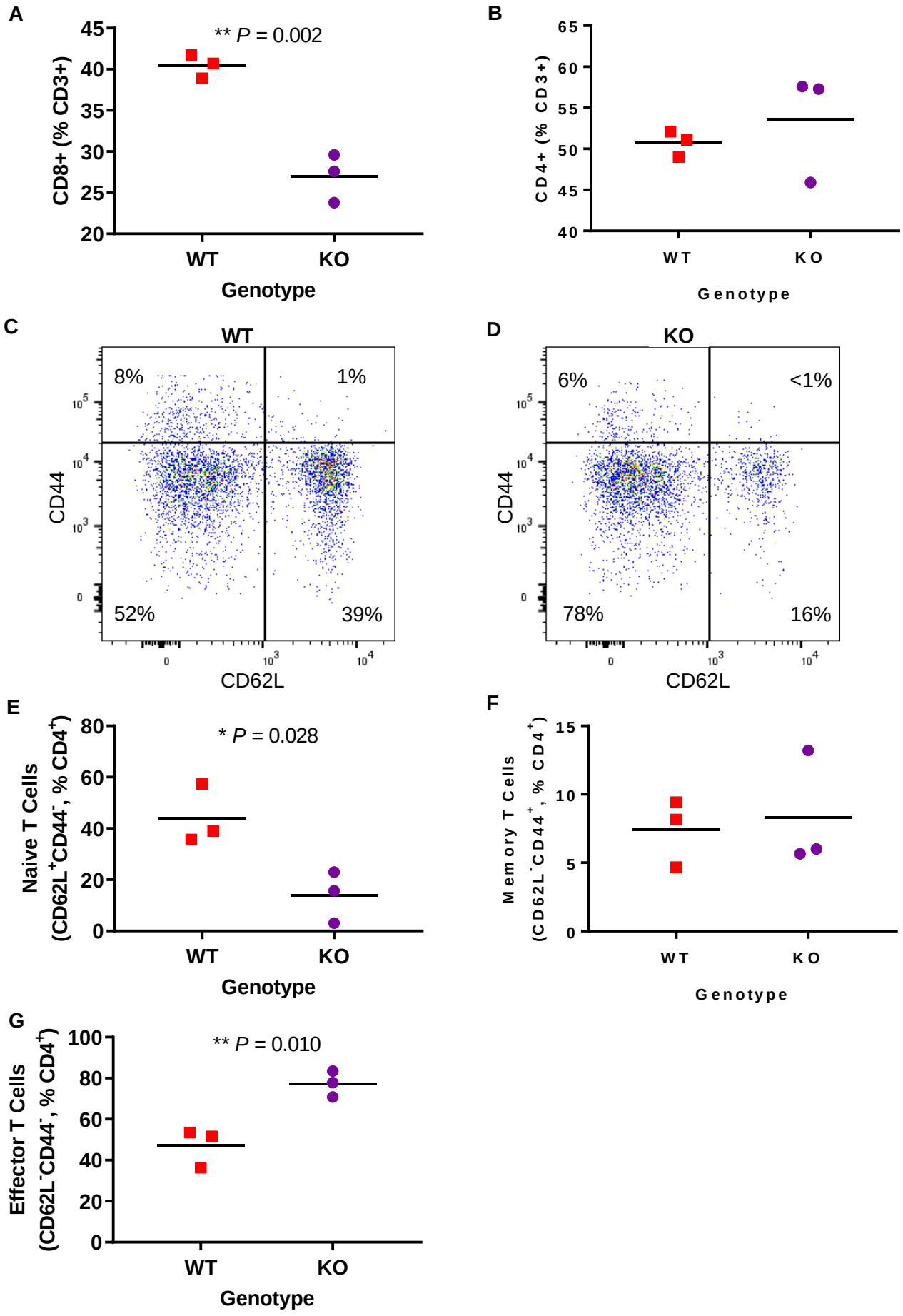

A
** P = 0.002
CD8+ (% CD3+)
45
40
35
30
25
20
WT
KO
Genotype
B
CD4+ (% CD3+)
65
60
55
50
45
40
WT
KO
Genotype
C
WT
8%
1%
52%
39%
CD44
CD62L
D
KO
6%
<1%
78%
16%
CD44
CD62L
E
* P = 0.028
Naive T Cells
(CD62L+CD44-, % CD4+)
80
60
40
20
0
WT
KO
Genotype
F
Memory T Cells
(CD62L-CD44+, % CD4+)
15
10
5
0
WT
KO
Genotype
G
** P = 0.010
Effector T Cells
(CD62L-CD44-, % CD4+)
100
80
60
40
20
0
WT
KO
Genotype

**Figure 38. T cell populations are altered in the draining lymph node from arthritic joints of Gal-1 KO mice.** Arthritis was induced in WT and Gal-1 KO mice by injection of arthritogenic (K/BxN) serum (i.p.) on days 0 and 2, disease progressed for four days and popliteal lymph nodes were collected to assess cellular infiltrate. Cells were isolated from lymph nodes and flow cytometry was used to analyse the cell populations. Percentages of T cell ($CD3^+$) subsets were determined as cytotoxic (CD8+) (**A**) and helper (CD4+) (**B**) T cells. Representative plots for both WT (**C**) and Gal-1 KO (**D**) show how expression of cell adhesion molecules (CD44 and CD62L) can further distinguish $CD4^+$ T cells into naïve ($CD62L^+CD44^-$) (**E**), effector ($CD62L^-CD44^-$) (**F**) and memory ($CD62L^-CD44^+$) (**G**) subsets. Statistical analysis was performed using an unpaired t-test with results showing the mean for each genotype. Results are considered significant when $P < 0.05$. n=3.

### *3.4.6. Inflammatory Mediator Profile*

Peripheral blood was taken from WT and Gal-1 KO mice on day 4. Serum was separated from whole blood, collected, and analysed by Luminex (Labospace Ltd. Milan) for levels of key inflammatory mediators. CXCL (KC) (figure 39A) and leukotriene B4 ($LTB_4$) levels (figure 39B) were measured by ELISA in serum of WT and Gal-1 KO mice. No significant differences were observed for either mediator between genotypes. IL-1β, IL-6, CCL2 (MCP-1) and TNF-α were also analysed, however results showed that levels were below the detectable range for the assay.

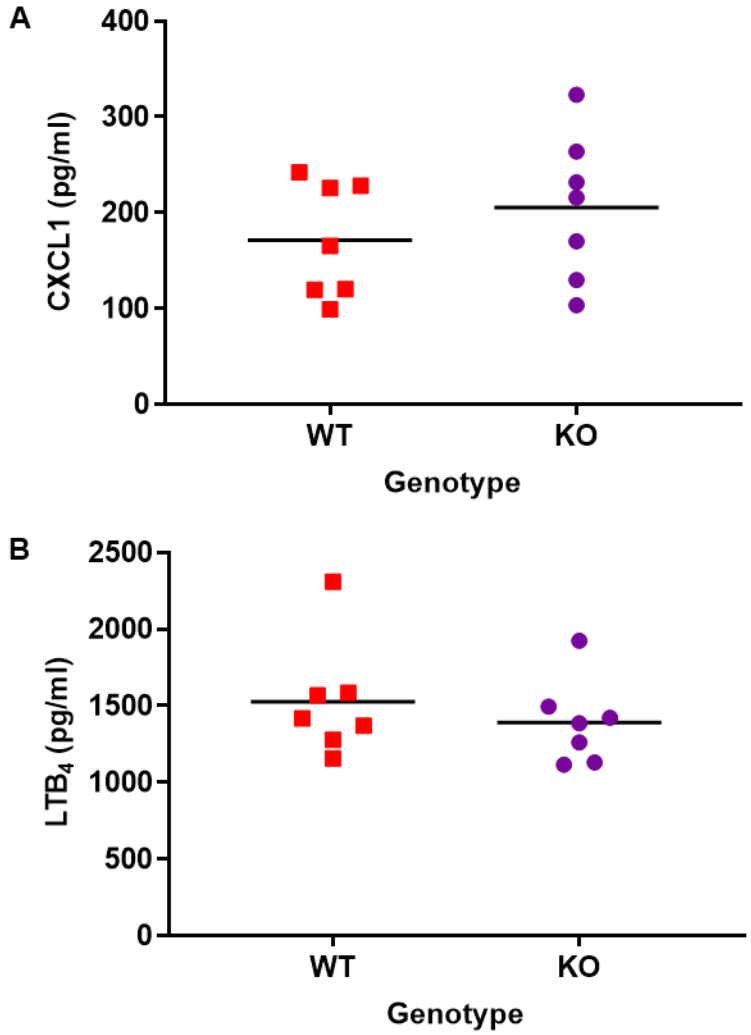



**Figure 39. The absence of Gal-1 had no effect on levels of inflammatory mediators in early arthritis.** Arthritis was induced in WT and Gal-1 KO mice by injection of arthritogenic (K/BxN) serum (i.p.) on days 0 and 2, disease progressed for four days and inflammatory mediator levels within the plasma were assessed. Plasma was separated from blood obtained by cardiac puncture and concentrations of CXCL1 (KC) (**A**) and Leukotriene B4 ($LTB_4$) (**B**) were measured by ELISA. Statistical analysis was performed using an unpaired t-test with results showing the mean for each genotype. Results considered significant if $P < 0.05$. n=7 mice per group.

### *3.4.7. Resolution of Inflammatory Arthritis*

A full disease profile for K/BxN serum transfer induced arthritis, was obtained for Gal-1 KO mice and WT mice to assess resolution. The initial development of arthritis was slower in Gal-1 KO than WT mice as indicated by the delay to reach peak disease (figure 40A) verifying the results observed in the previous 4 day experiment. A significant delay in disease resolution was observed for Gal-1 KO mice compared to their WT counterparts (figure 40A). Paw oedema was significantly lower in Gal-1 KO mice over the entire course of the disease (figure 40B). In line with the more rapid disease development observed in WT mice, they also lost significantly more weight during the first 4 days of observation (WT = 7.63% ± 1.28%, KO = 0.47% ± 1.04%; $p$ = 0.0004, figure 40C). Following an approximate 5% loss in body weight during peak arthritis, Gal-1 KO mice then maintained weight over the course of remission whereas WT mice began to regain weight, a result seen from day 25 onwards (figure 40C). No significant differences in disease incidence was observed between genotypes (figure 40D).

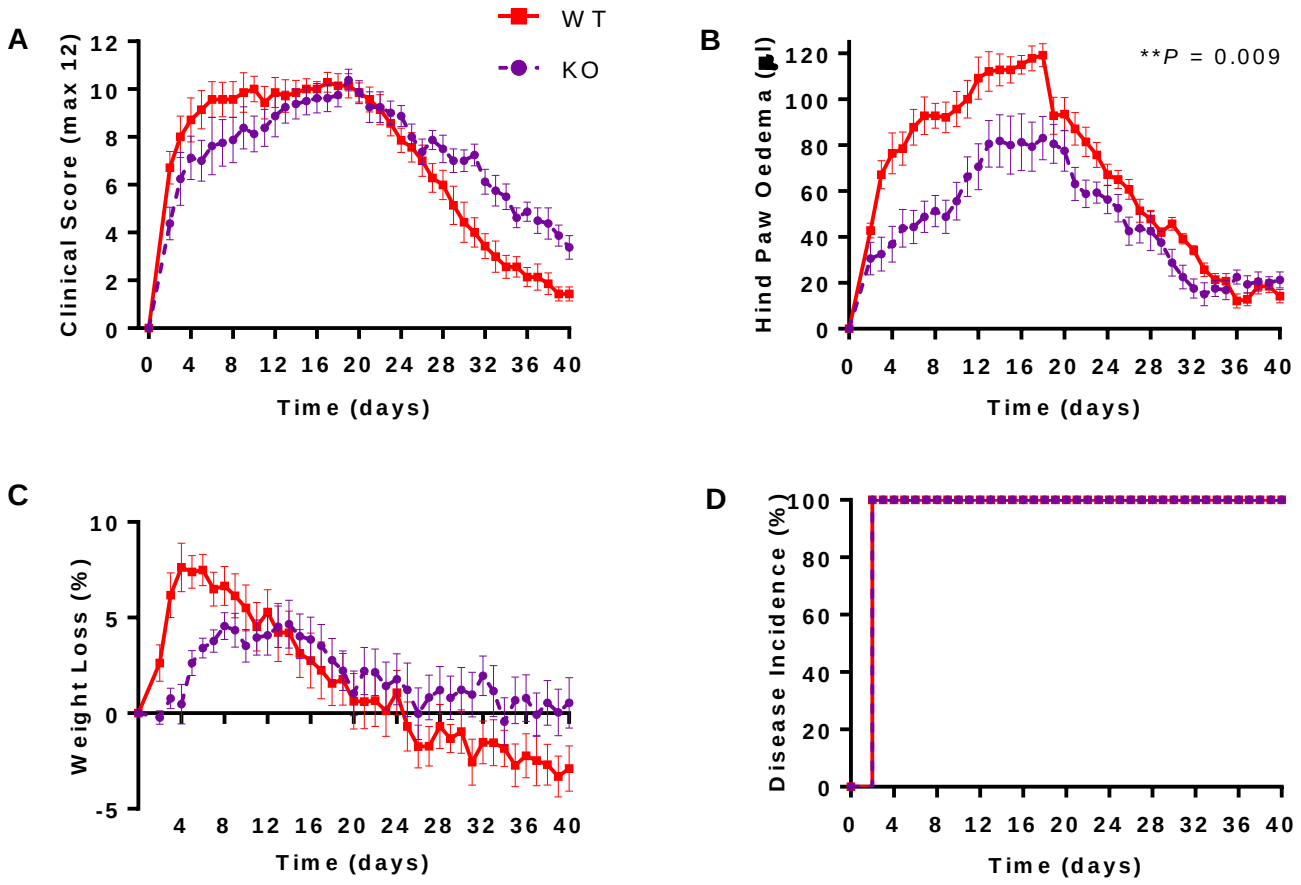



**Figure 40. Gal-1 KO mice display an altered arthritis profile.**
Arthritis was induced in WT and Gal-1 KO mice by injection of arthritogenic (K/BxN) serum (i.p.) on days 0 and 2. Disease severity was clinically assessed for 40 days post-induction by clinical scoring (**A**), paw oedema (**B**) and weight loss (**C**). Incidence of disease (**D**) was also recorded. Statistical analysis was performed using a Two-way RM ANOVA with results displayed showing the mean ± SEM, in all cases significant results are considered as $P < 0.05$. n=7-8.

### *3.4.7.1. Remission Indices*

Remission indices were calculated following the 40 days of observation, as displayed in figure 41. Values indicate the time points when mice reached the maximum magnitude of arthritis ($T_{max}$), when 50% of the maximum was reached ($T_{50}$) and the resolution interval (Ri) determined as the time from $T_{max}$ to $T_{50}$. Results for the two genotypes, show a delay in $T_{max}$ for the Gal-1 KO mice compared to their WT counterparts (19 days and 17 days, respectively) as well as a delay in $T_{50}$ (WT = 29 days and KO = 35 days). Consequently the $R_i$ value was increased in Gal-1 KO (16 days) compared to WT (12 days) mice, indicating that time to peak disease is lengthened in Gal-1 KO mice compared to WT and Gal-1 KO mice take longer to resolve again, resulting in their disease remission being a longer and slower process compared to WT mice (figure 41).

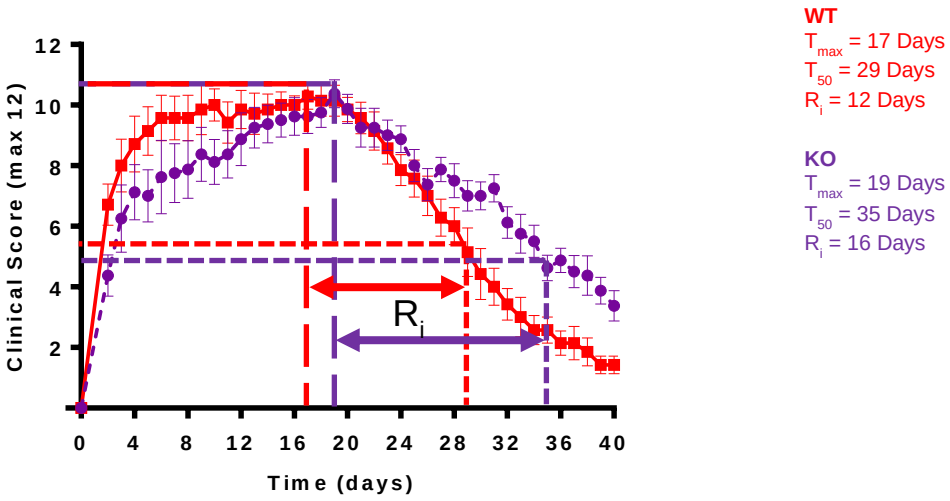



**Figure 41. Maximum magnitude and resolution of arthritis were delayed in Gal-1 KO mice.**
Arthritis was induced in WT and Gal-1 KO mice by injection of arthritogenic (K/BxN) serum (i.p.) on days 0 and 2. Mice were scored clinically for 40 days post-induction, and each swollen feature (wrist and ankle and respective pads and digits) was given a score of one, with a maximum score of 12 per mouse. Remission indices were calculated to indicate the time points when mice reached the maximum magnitude of arthritis ($T_{max}$), when 50% of the maximum was reached ($T_{50}$) and the resolution interval ($R_i$) the time from $T_{max}$ to $T_{50}$. n=7-8.

### *3.4.8. Joint Histology*

The resolution outcome was assessed in the previously inflamed joints from WT and Gal-1 KO mice in remission from arthritis. Microscopic damage was low in both genotypes at day 40 as would be expected. No significant differences were observed between genotypes (figure 42A-C).

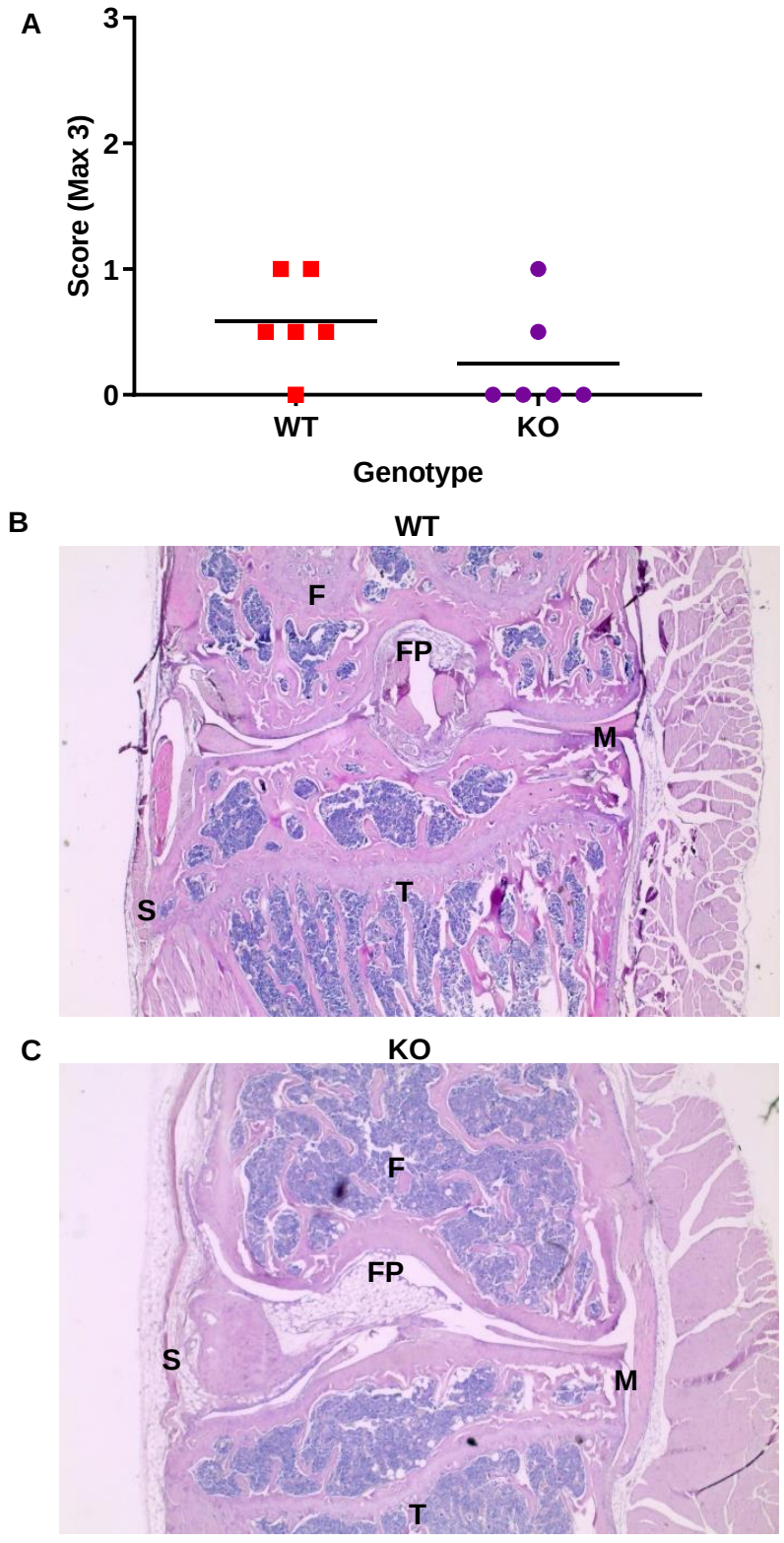



**Figure 42. The absence of Gal-1 did not modulate the outcome of arthritis remission as assessed microscopically.**
Arthritis was induced in WT and Gal-1 KO mice by injection of arthritogenic (K/BxN) serum (i.p.) on days 0 and 2. Disease progressed for forty days after which knee joints were collected for histology. H&E stained sections were analysed and scored blindly. Stained sections were scored on a scale of zero to three by assessing level of infiltrate and thickness of the synovium (A). Representative images for WT (B) and KO (C) show scores of 0.5 and 0 respectively. Statistical analysis was performed using an unpaired t-test with results showing the mean for each genotype, in all cases significant results are considered as $P < 0.05$. n=6.
# = leukocyte infiltrate, F = femur, FP = fat pad, M = meniscus, S = synovium, T = tibia.

### *3.5. The Effect of Exogenous Gal-1 in the Resolution of Inflammatory Arthritis.*

To explore the effect of exogenous hrGal-1 the K/BxN model of inflammatory arthritis was performed on WT mice administered hrGal-1 (3µg) or vehicle (20µl $DPBS^{+/+}$) by intraplantar injection (figure 43). Mice were treated on alternate days from established disease on day 3 and sacrificed during peak (day 7) or resolving (day 11) arthritis.

#### *3.5.1. Disease Severity*

Intraplantar treatment with hrGal-1 had no effect on either clinical score (figure 43A) or oedema (figure 43B).

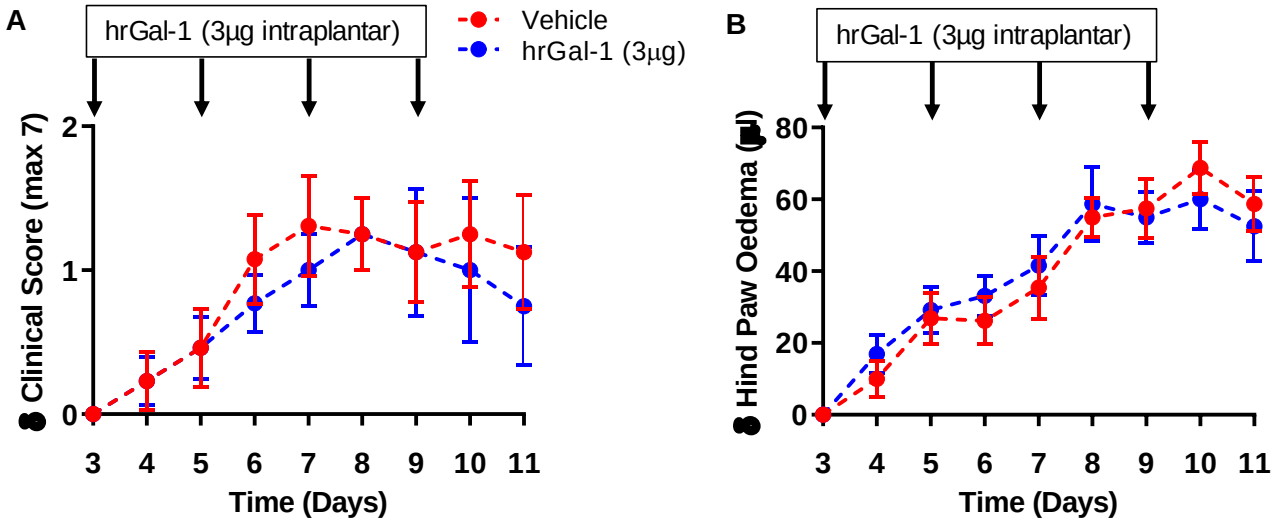



**Figure 43. Intraplantar administration of Gal-1 had no effect on arthritis in the hind paw.**
Arthritis was induced in WT mice by injection of arthritogenic (K/BxN) serum (i.p.) on days 0 and 2. Mice were then treated with hrGal-1 (3µg) or vehicle (20µl $DPBS^{+/+}$) by intraplantar injection on days 3, 5, 7 and 9. The effect of hrGal-1 was measured against contralateral vehicle control for clinical score (**A**) and hind paw oedema (**B**) and recorded as the difference from baseline (day 3). Statistical analysis was performed using a Two-way mixed effects ANOVA with results displayed showing the mean ± SEM, in all cases significant results are considered as $P < 0.05$. n=13 (days 0-7) or 8 (days 8-11) mice per group.

### *3.5.2. Leukocyte Infiltration*

During the resolving phase (day 11) there was no significant differences in any of the leukocyte subsets that had infiltrated hrGal-1 treated paws compared to vehicle controls as assessed by flow cytometry (figure 44A-D). However, data revealed a subtle trend towards a decreased percentage of neutrophils (figure 44A) and increased percentage of macrophages (figure 44C) in response to hrGal-1.

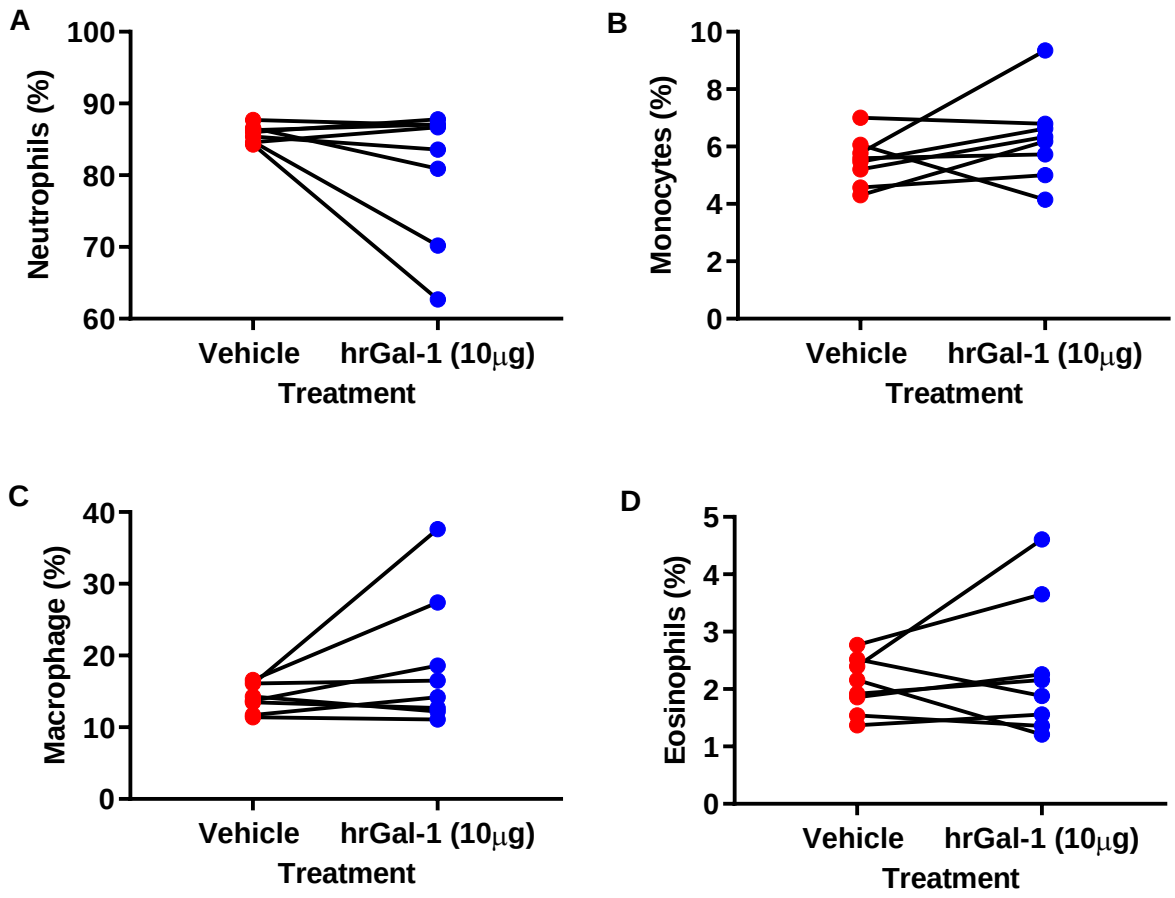



**Figure 44. Intraplantar administration of Gal-1 had no significant effect on leukocyte infiltration.**
Arthritis was induced in WT mice by injection of arthritogenic (K/BxN) serum (i.p.) on days 0 and 2. Mice were treated with hrGal-1 (3μg) or vehicle (20μl $DPBS^{+/+}$) by intraplantar injection on days 3, 5, 7 and 9. Disease progressed for 11 days and hind paws were collected and tissue digested. Cellular infiltrate was assessed by flow cytometry and percentage of neutrophils ($Ly6G^{+}$) (**A**), monocytes (Ly6C+) (**B**), macrophages (F4/80+) (**C**) and eosinophils (SiglecF+) (**D**) were calculated from the $CD45^{+}$ cells. Statistical analysis was performed using a paired t-test with results showing the individual values for treatment and vehicle control. Results considered significant if $P < 0.05$. n=8.

### *3.6. The Role of Endogenous Gal-1 in Efferocytosis.*

Efferocytosis was assessed by injecting fluorescently (CFSE) labelled apoptotic human neutrophils ($2.5 \times 10^6$ cells) into the peritoneal cavity of WT and Gal-1 KO mice. After 30 or 60 mins cells were collected from the cavity by peritoneal lavage and analysed by flow cytometry (figure 45).

Results, as shown in figure 45A revealed that Gal-1 KO mice had a significantly lower percentage of (F4/80+) macrophages (26.75% ± 2.5%) within the peritoneum compared to WT mice (41.43% ± 1.78%). This relatively decreased percentage of macrophages in Gal-1 KO mice did not confer a difference between genotypes in the percentage of macrophages positive for CFSE at either time point (figure 45B & C). Expectedly, there was an increase in percentages of macrophages CFSE+ from 30 mins (figure 45B) to 60 mins (figure 45C)

Assessment of the CFSE median fluorescence intensity (MFI) of the efferocytic (F4/80+CFSE+) population of cells showed that there was no difference between genotypes following a 30 min period of efferocytosis (figure 45D) indicating a similar number of apoptotic cells had been engulfed per macrophage for each genotype. Following 60 minutes of efferocytosis (figure 45E) Gal-1 KO mice were shown to have ingested more apoptotic neutrophils than their WT counterparts (6810 ± 480 vs 5730 ± 400), however this increase was not significant.

The percentage of CFSE+ apoptotic neutrophils remaining in the cavity was also analysed at both time points. When assessed as a percentage of the whole CFSE+

population the results were comparable between genotypes, with an expected decline in percentage of free CFSE+ apoptotic neutrophils at 60 mins (22.93% ± 0.69%) (figure 45G) compared to 30 mins (38.48% ± 5%) (figure 45F). However, a limitation of these values is that the percentage is relative to the other cells in the cavity, therefore actual number of apoptotic CFSE+ free neutrophils may be different between genotype.

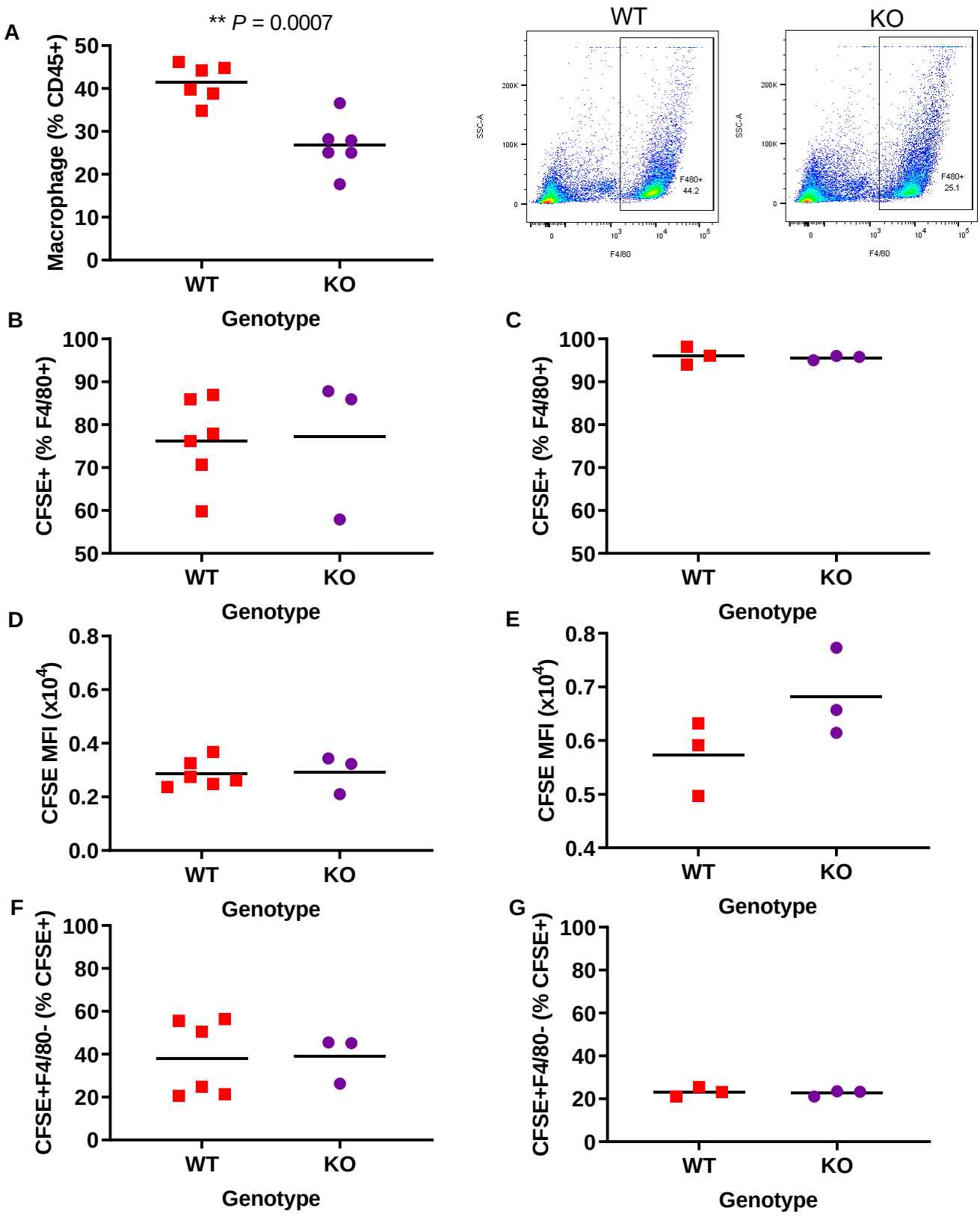

A
** P = 0.0007
Macrophage (% CD45+)
50
40
30
20
10
0
WT
KO
Genotype
WT
KO
SSC-A
200K
100K
0
F480+
44.2
F480+
25.1
F4/80
B
CFSE+ (% F4/80+)
100
90
80
70
60
50
C
D
CFSE MFI (x10^4)
0.8
0.6
0.4
0.2
0.0
E
0.7
0.5
F
CFSE+F4/80- (% CFSE+)
G

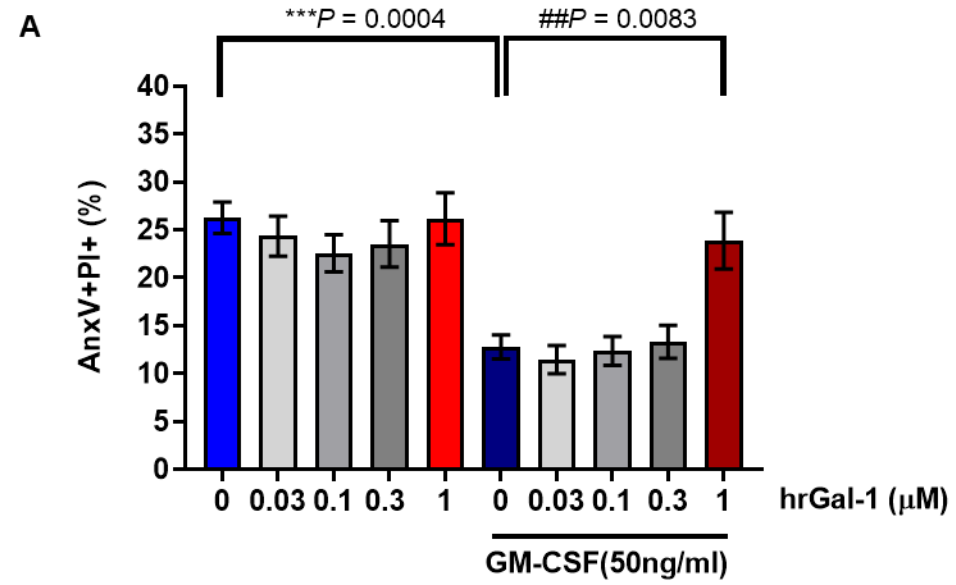
**Figure 45. In the absence of Gal-1 the percentage of macrophages were decreased following efferocytosis with no significant impact on apoptotic cell engulfment.**
CFSE labelled apoptotic neutrophils (2.5 x $10^6$ cells) were injected into the peritoneal cavity of Gal-1 KO and WT mice. After 30 or 60 mins cells were collected from the cavity by peritoneal lavage and stained with antibodies to detect murine hematopoietic cells (CD45), murine macrophages (F4/80) and human neutrophils (CD66b). The CD45+ peritoneal exudate was assessed for percentage of macrophages (F4/80+) with representative flow cytometry plots displayed (**A**) and the macrophage population analysed for percentage CFSE+ after 30 mins (**B**) and 60 mins (**C**). The CFSE median fluorescence intensity was determined for the double positive efferocytic population (F4/80+CFSE+) following 30 mins (**D**) and 60 mins **(E)**. The CFSE+ population was gated from the whole cell population and the percentage of free CFSE+ apoptotic neutrophils remaining within the cavity after 30 mins (**F**) or 60 mins (**G**) was determined as those F4/80-. Statistical analysis was performed using an unpaired t-test with results considered significant if $P < 0.05$. n=3-6.

### *3.7. The Effect of Gal-1 on Apoptosis of Human Neutrophils in vitro.*

Neutrophils isolated from human peripheral blood were treated ± hrGal-1 (0.03, 0.1, 0.3 and 1μM) ± GM-CSF (50ng/ml) or SAA (10μg/ml). Neutrophils were incubated overnight and levels of apoptosis were primarily determined by AnxV/PI using flow cytometry. Apoptosis was also quantified by mitochondrial membrane potential using $DiOC_6$ staining and by morphology from cytospins. Additionally, phosphorylation of intracellular signalling proteins AKT, ERK and p38 MAPK, involved in the neutrophil apoptosis pathway, were also investigated by Western blot. Neutrophil release of AnxA1 was also detected by Western blot and expression analysed by flow cytometry. Early PS exposure on neutrophils in response to hrGal-1 was also assessed by AnxV/PI staining at 1h and 4h (± GM-CSF (50ng/ml)) timepoints.

#### *3.7.1. AnxV/PI*

Human PMN were isolated and incubated overnight to assess the impact of hrGal-1 on spontaneous apoptosis. Treatment with hrGal-1 alone did not modulate the level of late apoptosis in human neutrophils. As expected GM-CSF significantly reduced the spontaneous apoptosis of neutrophils. Treatment with hrGal-1 in addition to GM-CSF reversed the protective effect of GM-CSF resulting in significantly increased levels of apoptosis (figure 46A & B). Results for the other stages of neutrophil apoptosis are summarised in table 12 as percentages (mean ± SEM) of the neutrophil populations found within each of the cell viability quadrants. The significant differences in late apoptosis are complemented by differences in the percentage of viable neutrophils.


A
***P = 0.0004
##P = 0.0083
AnxV+PI+ (%)
40
35
30
25
20
15
10
5
0
0 0.03 0.1 0.3 1 0 0.03 0.1 0.3 1
hrGal-1 (μM)
GM-CSF(50ng/ml)

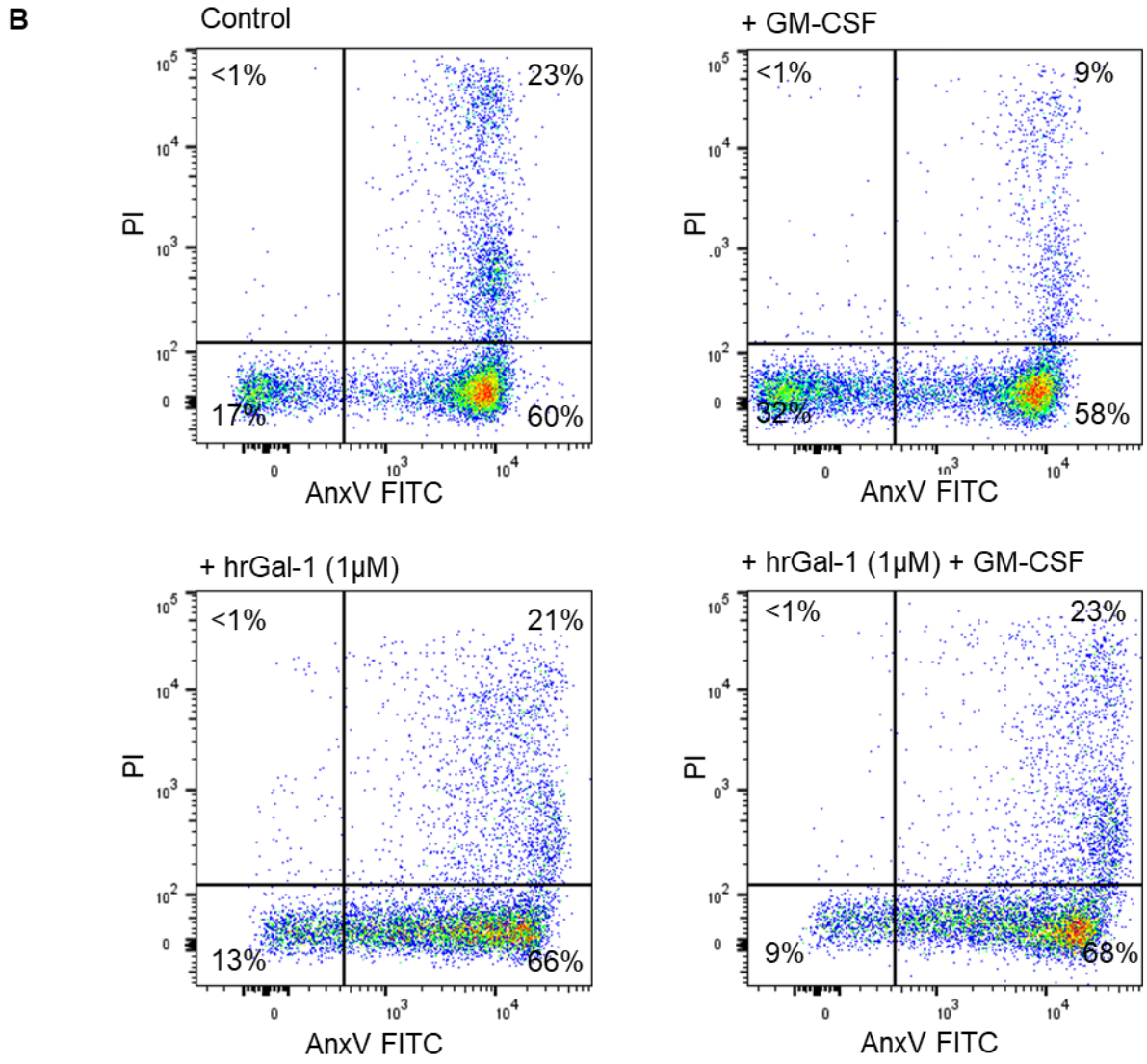

B
Control
<1%
23%
17%
60%
+ GM-CSF
<1%
9%
32%
58%
+ hrGal-1 (1μM)
<1%
21%
13%
66%
+ hrGal-1 (1μM) + GM-CSF
<1%
23%
9%
68%
PI
AnxV FITC

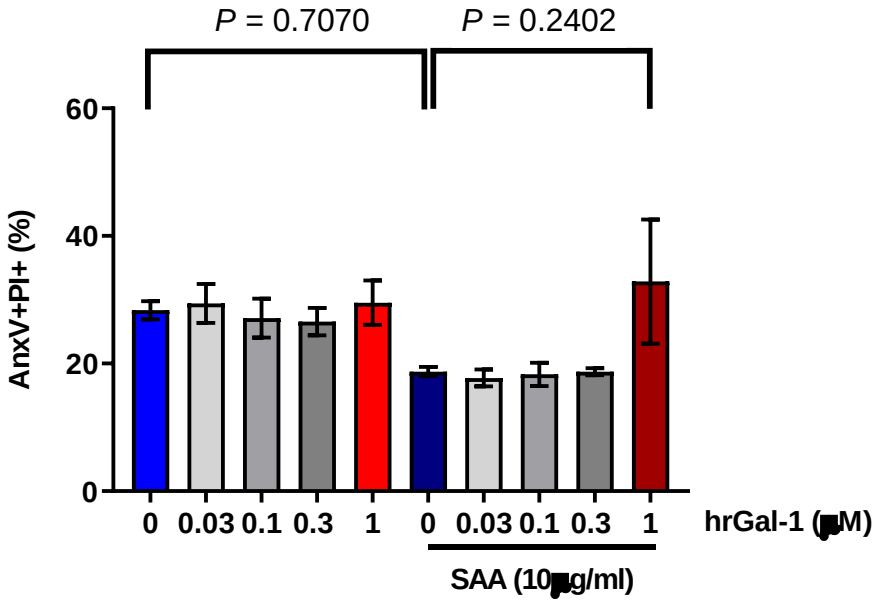
**Figure 46. Gal-1 induces apoptosis of GM-CSF stimulated human neutrophils.**
Neutrophils isolated from human peripheral blood were incubated (20h) in RPMI + 5% autologous serum ± GM-CSF (50ng/ml) ± hrGal-1 (0.03, 0.1, 0.3 or 1µM). Levels of neutrophil apoptosis were assessed by flow cytometry using AnxV and Propidium Iodide (PI). A quadrant gating strategy was applied to determine viable ($AnxV^-PI^-$), early apoptotic ($AnxV^+PI^-$), late apoptotic ($AnxV^+PI^+$) and necrotic ($AnxV^-PI^+$) neutrophil populations. The percentage of double positive ($AnxV^+PI^+$) neutrophils is shown for each treatment as the mean ± SEM (**A**) together with representative flow plots (**B**). Statistical analysis was performed using a one-way ANOVA and in all cases results are considered significant when $P < 0.05$. * = significant difference between control and GM-CSF (50ng/ml) treatment. # = significant difference between GM-CSF (50ng/ml) and hrGal-1µM + GM-CSF (50ng/ml) treatment. n=10.

## Table 12. Gal-1 induces apoptosis of GM-CSF stimulated human neutrophils.

| Treatment | Viable | Early Apoptotic | Late Apoptotic | Necrotic |
|---|---|---|---|---|
| Control | 16.53 ± 2.62 | 57.32 ± 2.81 | 26.28 ± 1.65 | 0.38 ± 0.12 |
| hrGal-1 (1µM) | 10.63 ± 1.91 | 59.63 ± 3.04 | 26.18 ± 2.17 | 0.51 ± 0.22 |
| GM-CSF (50ng/ml) | 32.72 ± 3.87 | 54.56 ± 3.05 | 12.77 ± 1.26 | 0.59 ± 0.12 |
| GM-CSF (50ng/ml) + hrGal-1 (1µM) | 13.78 ± 2.61 | 61.82 ± 3.39 | 23.89 ± 2.97 | 0.41 ± 0.09 |

SAA (10µg/ml) was also used as an alternative pro-survival factor to GM-CSF, and the results revealed a similar trend. The proportion of neutrophils undergoing spontaneous apoptosis with SAA was reversed by addition of hrGal-1 (figure 47A & B), however, the results were not statistically significant. Again, results for the other stages of neutrophil apoptosis are summarised in table 13. Although statistical significance was not reached for the data, there is a clear trend for increased AnxV+PI+ late apoptotic percentages in SAA treated neutrophils co-incubated with hrGal-1 (18.73% ± 0.73% vs 32.87% ± 9.72%). As expected, the percentage of the population deemed as viable showed the inverse with decreased AnxV-PI- cells seen in response to addition of hrGal-1 also as opposed to SAA alone (4.48% ± 0.52% vs 21.8% ± 4.70%).

A


P = 0.7070
P = 0.2402
AnxV+PI+ (%)
60
40
20
0
0 0.03 0.1 0.3 1 0 0.03 0.1 0.3 1
hrGal-1 (μM)
SAA (10 μg/ml)

B

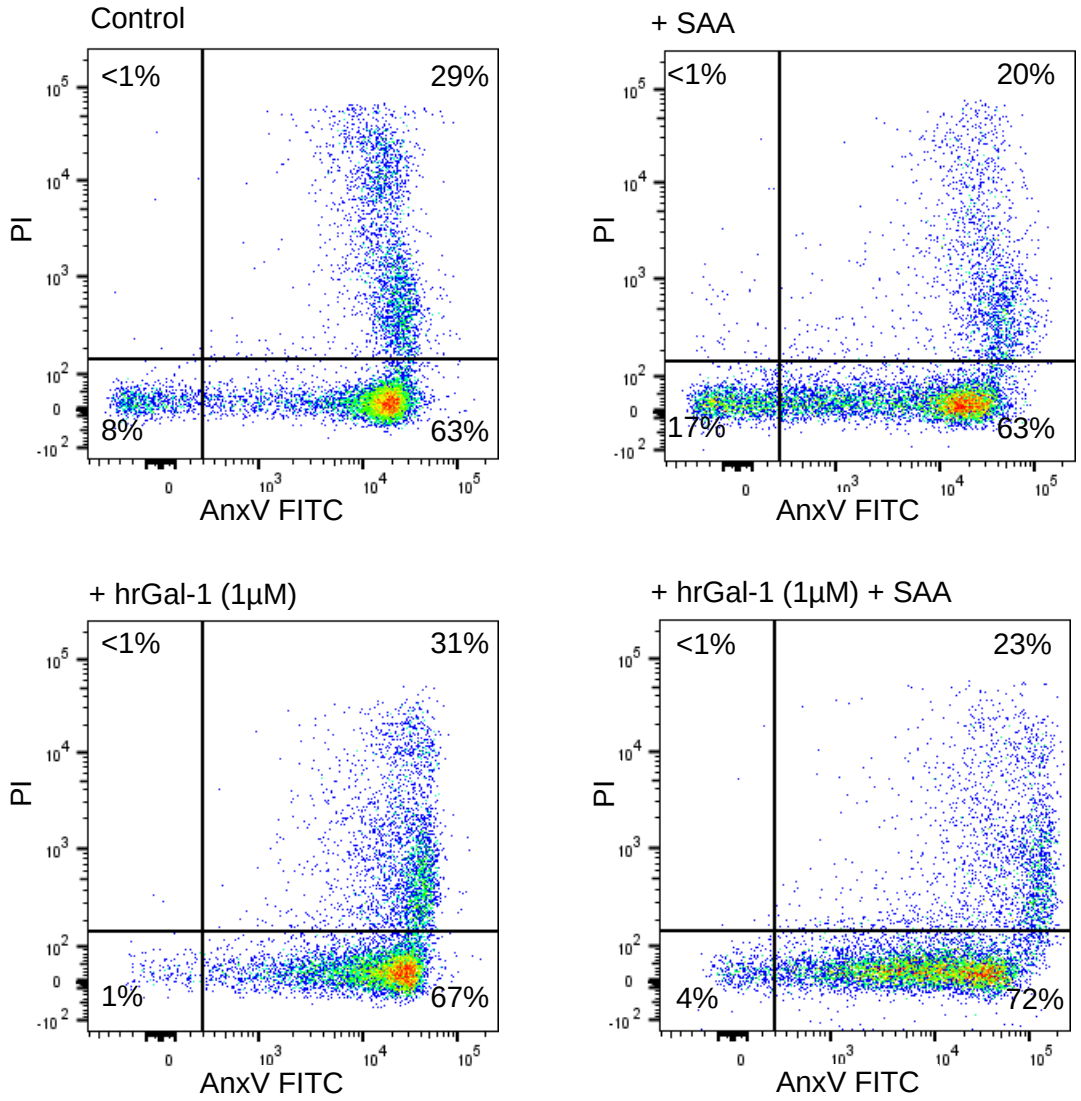

Control
<1%
29%
8%
63%
PI
AnxV FITC
+ SAA
<1%
20%
17%
63%
PI
AnxV FITC
+ hrGal-1 (1μM)
<1%
31%
1%
67%
PI
AnxV FITC
+ hrGal-1 (1μM) + SAA
<1%
23%
4%
72%
PI
AnxV FITC

**Figure 47. Gal-1 induces apoptosis of SAA stimulated human neutrophils.**
Neutrophils were isolated from the peripheral blood of healthy volunteers and incubated (20h) in RPMI – 1640 Medium + L-glutamine + 5% autologous serum ± SAA (10µg/ml) ± hrGal-1 (0.03, 0.1, 0.3 or 1µM). Levels of neutrophil apoptosis were assessed by flow cytometry using AnxV and Propidium Iodide (PI). A quadrant gating strategy was applied to determine viable ($AnxV^-PI^-$), early apoptotic ($AnxV^+PI^-$), late apoptotic ($AnxV^+PI^+$) and necrotic ($AnxV^-PI^+$) neutrophil populations. The percentage of double positive ($AnxV^+PI^+$) neutrophils is shown for each treatment as the mean ± SEM (**A**) with representative flow plots (**B**). Statistical analysis was performed using a one-way ANOVA and in all cases results are considered significant when $P < 0.05$. n=3.

## Table 13. Gal-1 induces apoptosis of SAA stimulated human neutrophils.

| Treatment | Viable | Early Apoptotic | Late Apoptotic | Necrotic |
|---|---|---|---|---|
| Control | 8.75 ± 3.36 | 62.77 ± 1.83 | 28.37 ± 1.43 | 0.15 ± 0.02 |
| hrGal-1 (1µM) | 3.18 ± 0.90 | 67.17 ± 3.61 | 29.53 ± 3.47 | 0.09 ± 0.02 |
| SAA (10µg/ml) | 21.8 ± 4.70 | 58.97 ± 4.14 | 18.73 ± 0.73 | 0.42 ± 0.09 |
| SAA (10µg/ml) + hrGal-1 (1µM) | 4.48 ± 0.52 | 62.57 ± 9.44 | 32.87 ± 9.72 | 0.09 ± 0.02 |

### *3.7.2. Nuclear Morphology*

For confirmation of apoptosis it is best practise to use more than one method of assessment. Here, AnxV/PI results were validated using cytospins to assess neutrophil morphology.

Cytospins were prepared for apoptotic neutrophils ± GM-CSF (50ng/ml) or SAA (10µg/ml) ± hrGal-1 (1µM) and stained with Kwik-Diff. From each cytospin 200 cells were counted and apoptotic neutrophils expressed as a percentage of the total neutrophil count. In line with the results obtained from the flow cytometry analysis morphological assessment of the nuclei revealed a decreased percentage of apoptosis in neutrophils treated with GM-CSF compared to untreated cells (30.14% ± 3.47% vs 56.21% ± 6.20%). This pro-survival was reversed by the addition of hrGal-1, which increased the percentage of apoptotic neutrophils (45.36% ± 5.48%), as displayed in figure 48 and table 14.

When SAA was used as the pro-survival factor in place of GM-CSF a similar trend was observed. As shown in figure 49, SAA significantly decreased the percentage of apoptotic neutrophils compared to control (25.50% ± 10.10% vs 59.63% ± 9.75%), an effect that was partially reversed by the addition of hrGal-1 (36.38% ± 1.65%) (see table 15).

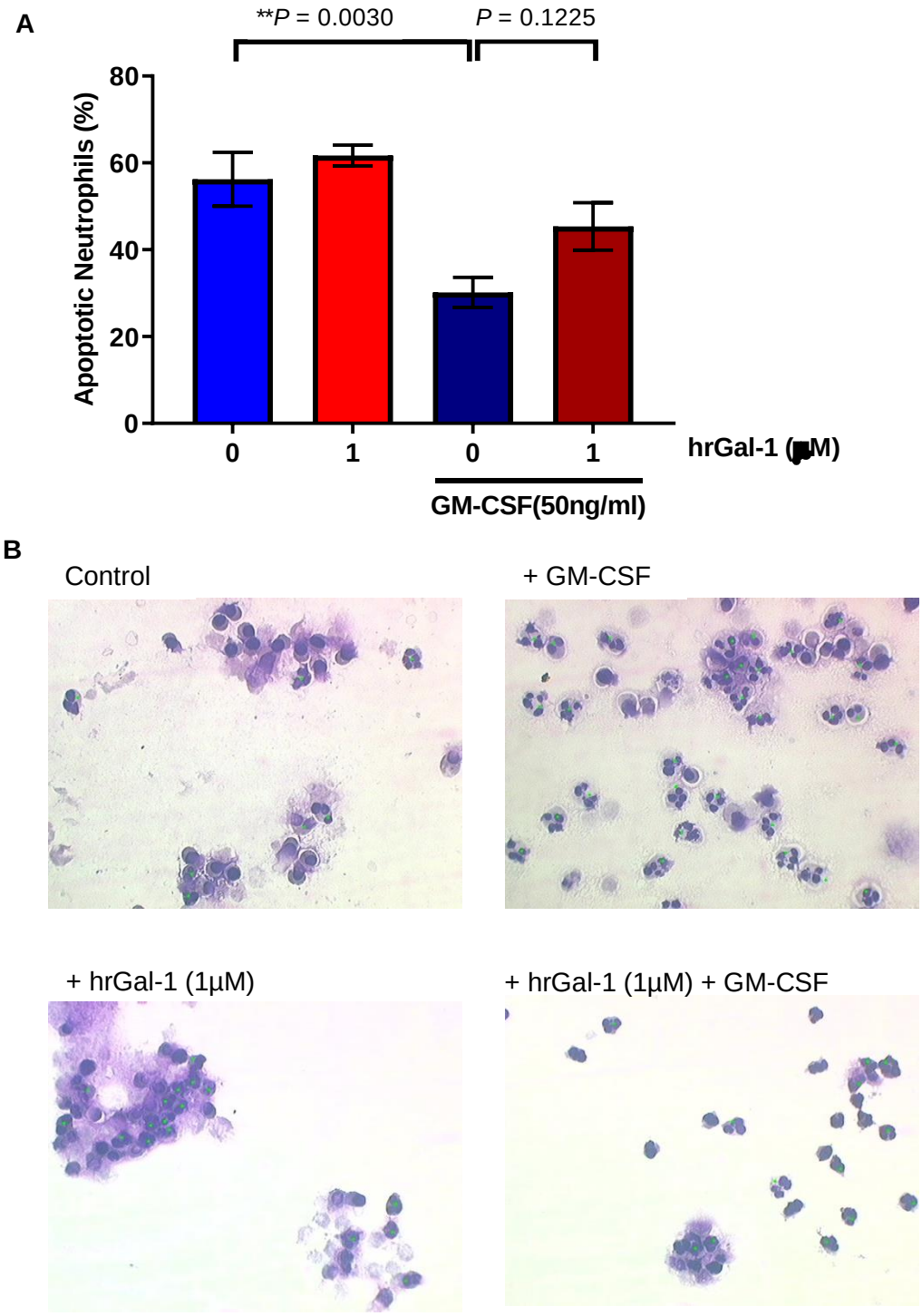



**Figure 48. Gal-1 reverses GM-CSF elicited survival of human neutrophils.**
Neutrophils isolated from human peripheral blood were incubated (20h) in RPMI – 1640 Medium + L-glut + 5% autologous serum ± GM-CSF (50ng/ml) ± hrGal-1 (1μM). Levels of neutrophil apoptosis were assessed by nuclear morphology using light microscopy. 200 cells were counted for each sample and apoptotic neutrophils expressed as a percentage. Cumulative data are shown as the mean ± SEM (**A**). Representative images are shown for each condition (**B**), with viable neutrophils indicated with a green spot. Statistical analysis was performed using a one-way ANOVA and in all cases results are considered significant when $P < 0.05$. n=7.

**Table 14. Gal-1 reverses GM-CSF elicited survival of human neutrophils.**

| Treatment | Viable | Apoptotic | SEM |
|---|---|---|---|
| Control | 43.79 | 56.21 | ± 6.20 |
| hrGal-1 (1µM) | 38.29 | 61.71 | ± 2.40 |
| GM-CSF (50ng/ml) | 69.86 | 30.14 | ± 3.47 |
| GM-CSF (50ng/ml) + hrGal-1 (1µM) | 54.64 | 45.36 | ± 5.48 |

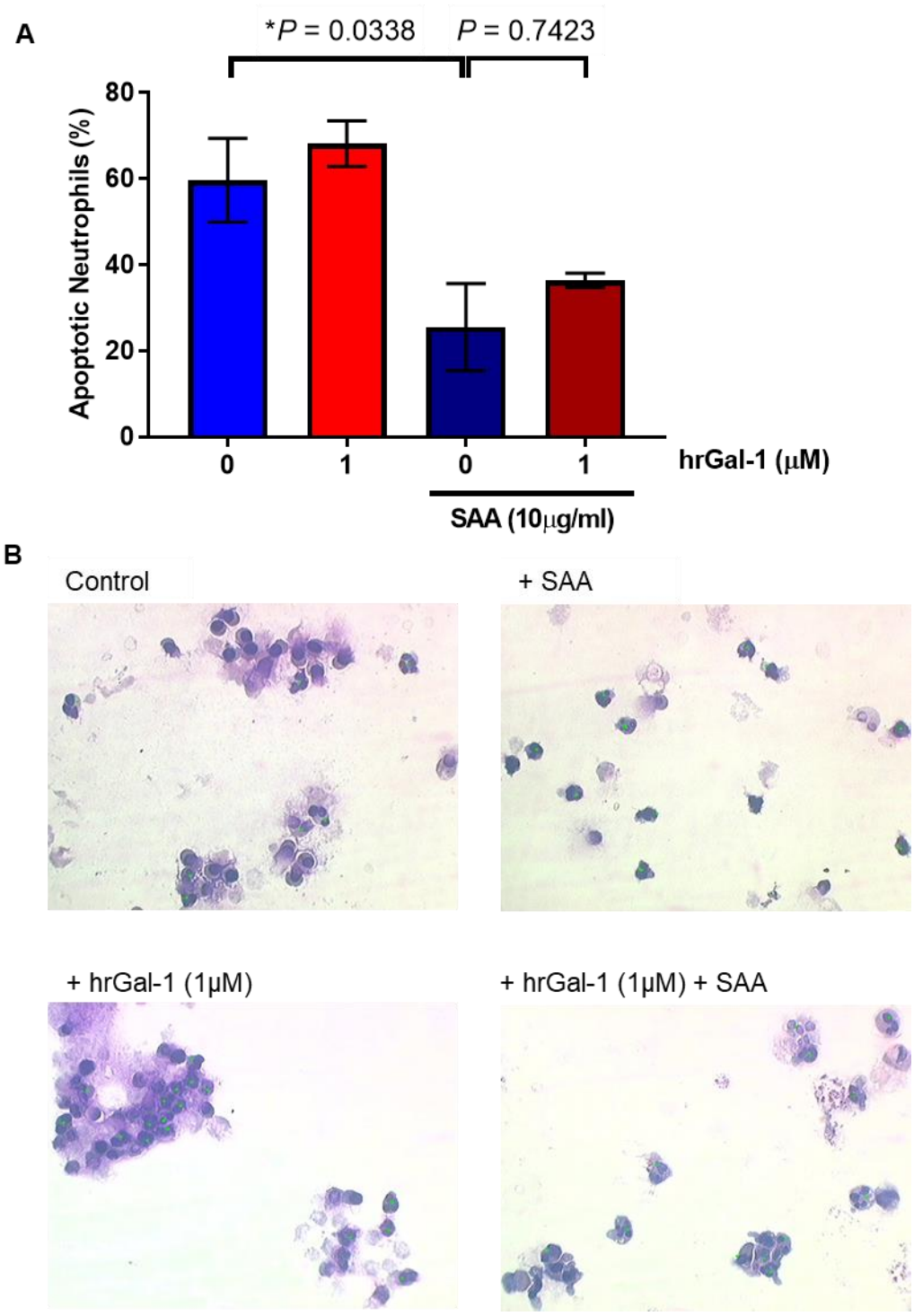



**Figure 49. Gal-1 reverses SAA elicited survival of human neutrophils.**
Neutrophils were isolated from the peripheral blood of healthy volunteers and incubated (20h) in RPMI – 1640 Medium + L-glut + 5% autologous serum ± SAA (10μg/ml) ± hrGal-1 (1μM). Levels of neutrophil apoptosis were assessed by nuclear morphology using light microspcopy. 200 cells were counted for each sample and apoptotic neutrophils expressed as a percentage. Cumulative data are shown as the mean ± SEM (**A**). Representative images are shown for each condition (**B**), with viable neutrophils indicated with a green spot. Statistical analysis was performed using a one-way ANOVA and in all cases results are considered significant when $P < 0.05$. n=4.

**Table 15. Gal-1 reverses SAA elicited survival of human neutrophils.**

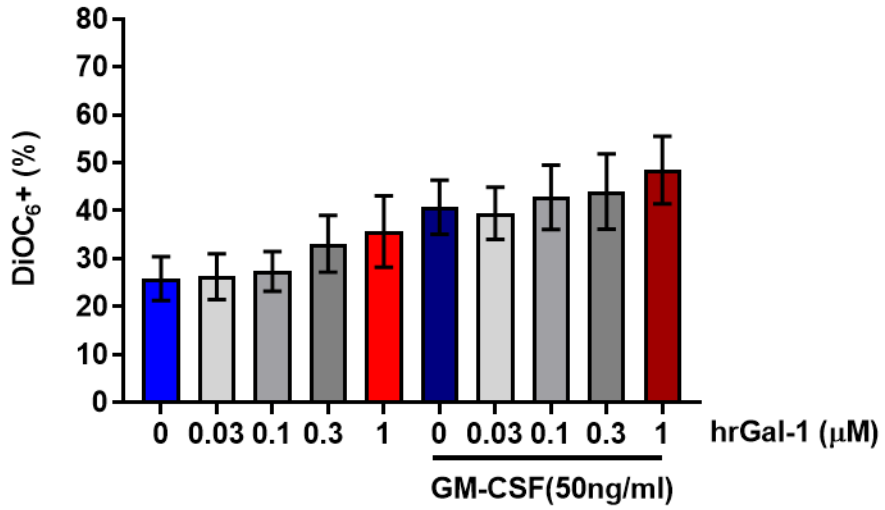
| Treatment | Viable | Apoptotic | SEM |
|---|---|---|---|
| Control | 40.38 | 59.63 | ± 9.75 |
| hrGal-1 (1µM) | 31.88 | 68.13 | ± 5.32 |
| SAA (10µg/ml) | 74.50 | 25.50 | ± 10.10 |
| SAA (10µg/ml) + hrGal-1 (1µM) | 63.63 | 36.38 | ± 1.65 |

### *3.7.3. $DiOC_6$*

Besides PS exposure another early event as neutrophils undergo apoptosis is the disruption to mitochondrial membrane potential thus apoptosis was also assessed by $DiOC_6$, a lipophilic dye that is selective for the mitochondria of live cells. Viable cells are DiOC6 high due to their density of lipids in their intact mitochondrial membrane, whereas apoptotic cells with their loss of membrane integrity stain with a relatively lower intensity. In this manner levels of apoptosis of neutrophils ± GM-CSF (50ng/ml) ± hrGal-1 (1µM) was determined by gating on the viable ($DiOC_6$ high) population of cells.

Results in table 16 are expressed as a percentage of the total single cell population ± SEM and displayed in figure 50. Apoptotic neutrophils had the lowest $DiOC_6$ staining and thus the least viable cells (25.82% ± 4.61%). Interestingly hrGal-1 treated neutrophils and GM-CSF treated neutrophils had an increased percentage of $DiOC_6$ positive cells (35.66% ± 7.42% and 40.73% ± 5.67% respectively) compared to control. Neutrophils that had been co-treated with both hrGal-1 and GM-CSF whilst undergoing spontaneous apoptosis had the highest population of DiOC6 positive cells (48.48% ± 7.04%), however these differences were not statistically significant.

A


80
70
60
50
40
30
20
10
0
DiOC6+ (%)
0 0.03 0.1 0.3 1 0 0.03 0.1 0.3 1
hrGal-1 (μM)
GM-CSF(50ng/ml)

B

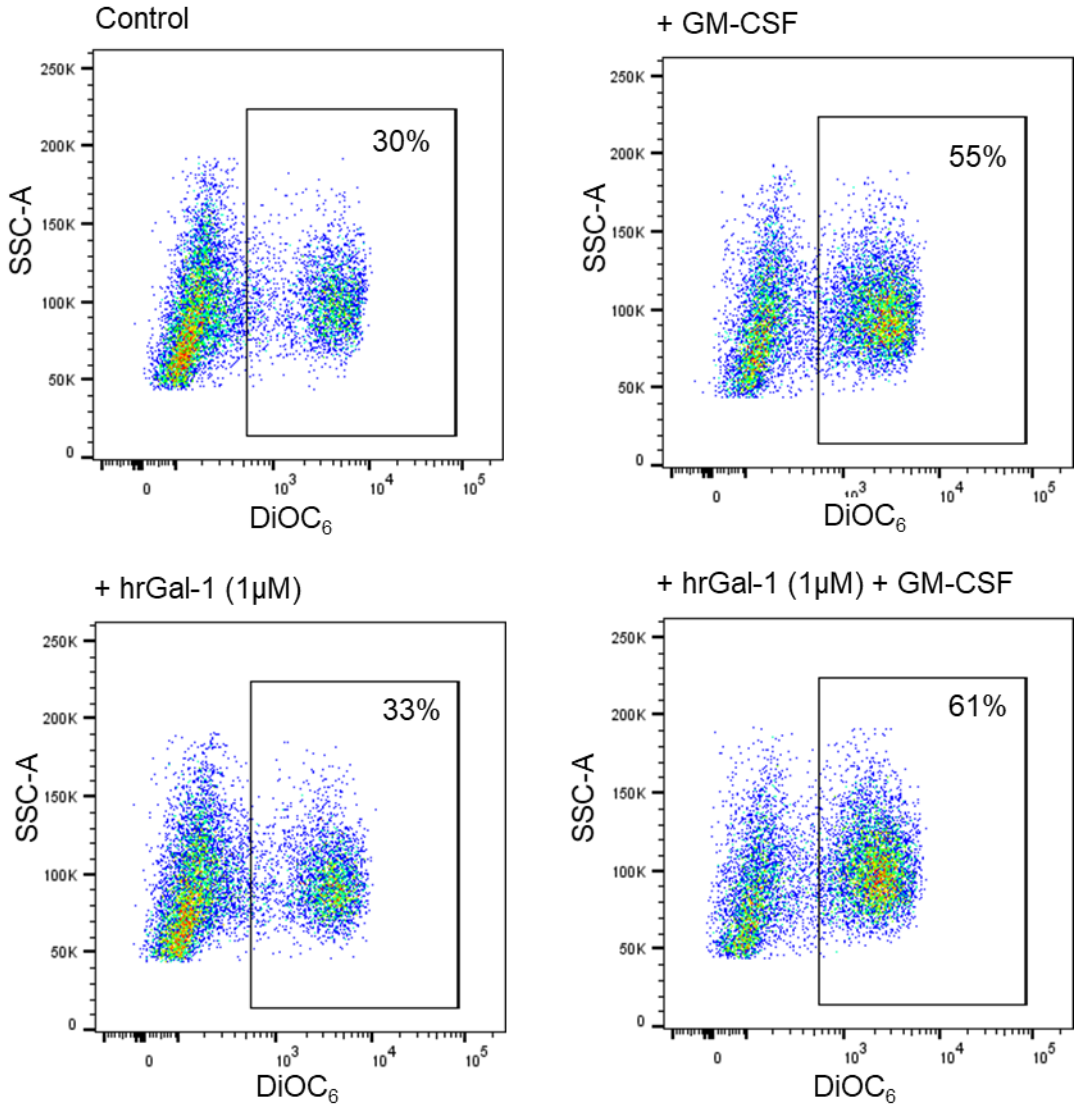

Control
30%
+ GM-CSF
55%
+ hrGal-1 (1μM)
33%
+ hrGal-1 (1μM) + GM-CSF
61%
SSC-A
DiOC6
250K
200K
150K
100K
50K
0
10^3
10^4
10^5

**Figure 50. Gal-1 has no significant effect on mitochondrial membrane potential during apoptosis.**
Neutrophils isolated from human peripheral blood were incubated (20h) in RPMI – 1640 Medium + L-glut + 5% autologous serum ± GM-CSF (50ng/ml) ± hrGal-1 (0.03, 0.1, 0.3 or 1μM). Mitochondrial membrane potential was assessed by $DiOC_6$ staining and analysed by flow cytometry. $DiOC_6$ positive neutrophils were gated from the single cell population and cumulative data is shown as the mean ± SEM (**A**) with representative flow plots (**B**) for each treatment. Statistical analysis was performed using a one-way ANOVA and in all cases results are considered significant when $P < 0.05$. n=9.

## Table 16. Gal-1 has no significant effect on mitochondrial membrane potential during apoptosis.

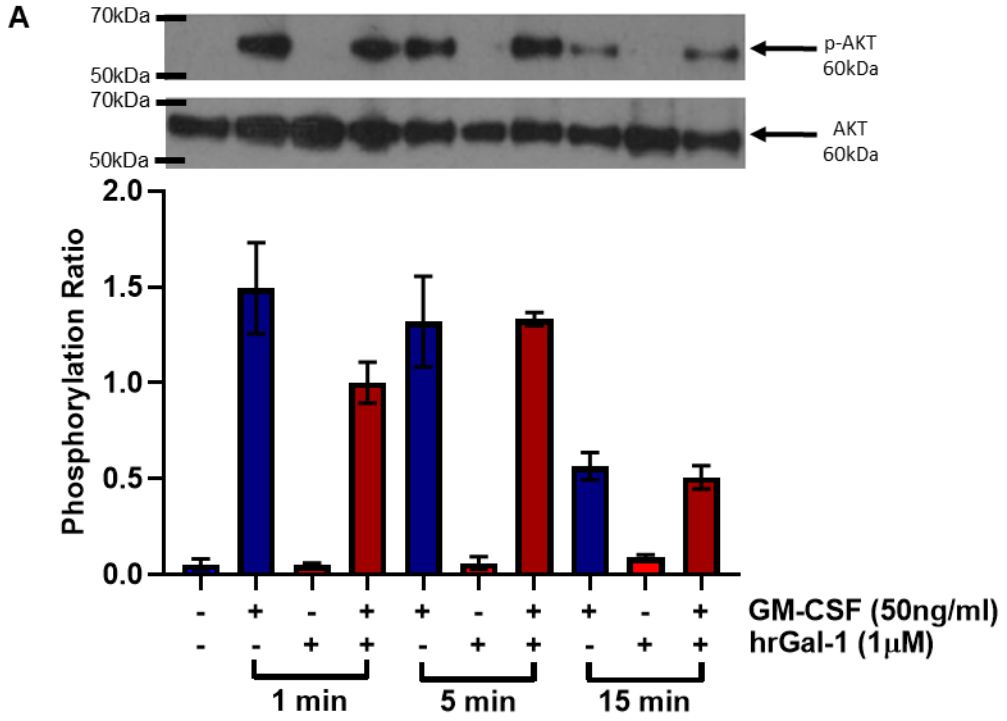
| Treatment | Viable | Apoptotic | SEM |
|---|---|---|---|
| Control | 25.82 | 74.18 | ± 4.61 |
| hrGal-1 (1μM) | 35.66 | 64.34 | ± 7.42 |
| GM-CSF (50ng/ml) | 40.73 | 59.27 | ± 5.67 |
| GM-CSF (50ng/ml) + hrGal-1 (1μM) | 48.48 | 51.52 | ± 7.04 |

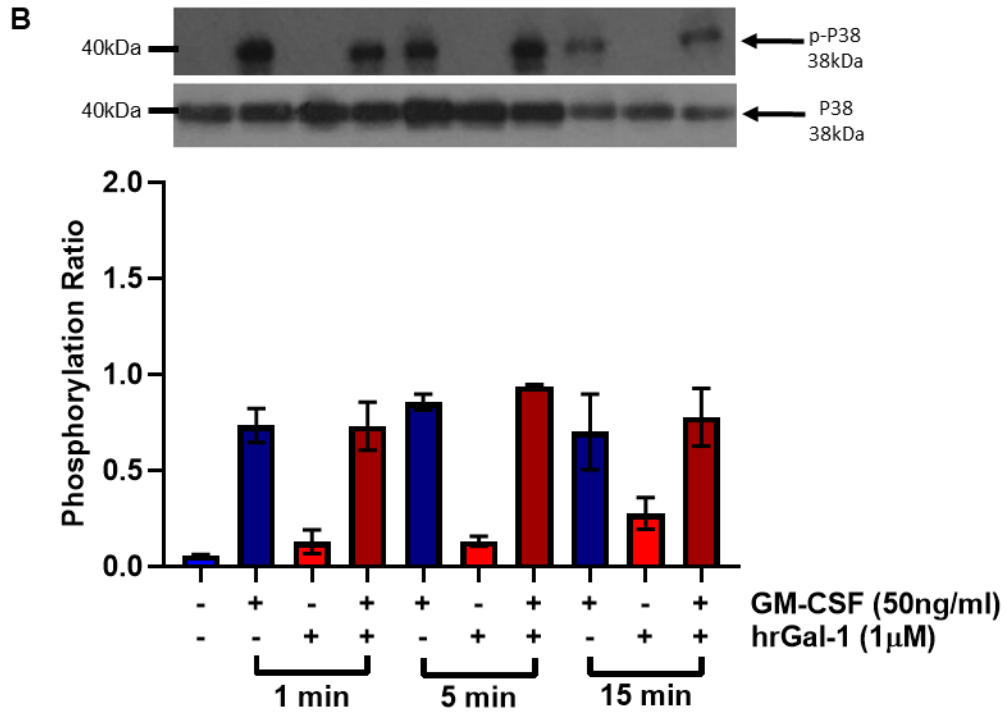
### *3.7.4. Intracellular Signalling*

Intracellular signalling pathways known to be involved in neutrophil apoptosis were investigated by Western blot. Results indicated that GM-CSF (50ng/ml) induced rapid phosphorylation of AKT within 1 minute. This response was mainly upheld at 5mins and reduced by 15mins, as displayed in figure 51A. HrGal-1 (1μM) in combination with GM-CSF shows a trend for decreased signalling at 1min whereas at 5mins and 15mins the phosphorylation of AKT was no different to GM-CSF only. The initial peak in GM-CSF induced phosphorylation of AKT was blunted by hrGal-1, resulting in comparably increased phosphorylation occurring at 5mins. No detectable phosphorylation of AKT occurred in response to stimulation with hrGal-1 alone, with all samples remaining comparable to unstimulated neutrophils.

Phosphorylation of p38 MAPK was also rapidly induced by GM-CSF at 1 min and remained relatively constant, as shown in figure 51B. The p38 MAPK signalling pathway was largely unchanged when neutrophils were co-treated with hrGal-1 in addition to GM-CSF. Neutrophils stimulated with hrGal-1 alone showed no significant signalling induction however phosphorylation of p38 MAPK was marginally enhanced at 15mins compared to unstimulated neutrophils.

The induction of ERK phosphorylation by GM-CSF stimulation commenced after 1min, and continued to increase at 5mins and 15mins, as in figure 51C. The signal induction was initially delayed when neutrophils were co-stimulated with hrGal-1, before increasing with longer stimulation, with highest phosphorylation levels at 15mins. Treatment with hrGal-1 alone induced a very subtle signalling response in neutrophils at 5mins and 15mins compared to unstimulated cells.


A
70kDa
50kDa
70kDa
50kDa
p-AKT
60kDa
AKT
60kDa
Phosphorylation Ratio
2.0
1.5
1.0
0.5
0.0
GM-CSF (50ng/ml)
hrGal-1 (1μM)
1 min
5 min
15 min


B
40kDa
40kDa
p-P38
38kDa
P38
38kDa
Phosphorylation Ratio
2.0
1.5
1.0
0.5
0.0
GM-CSF (50ng/ml)
hrGal-1 (1μM)
1 min
5 min
15 min

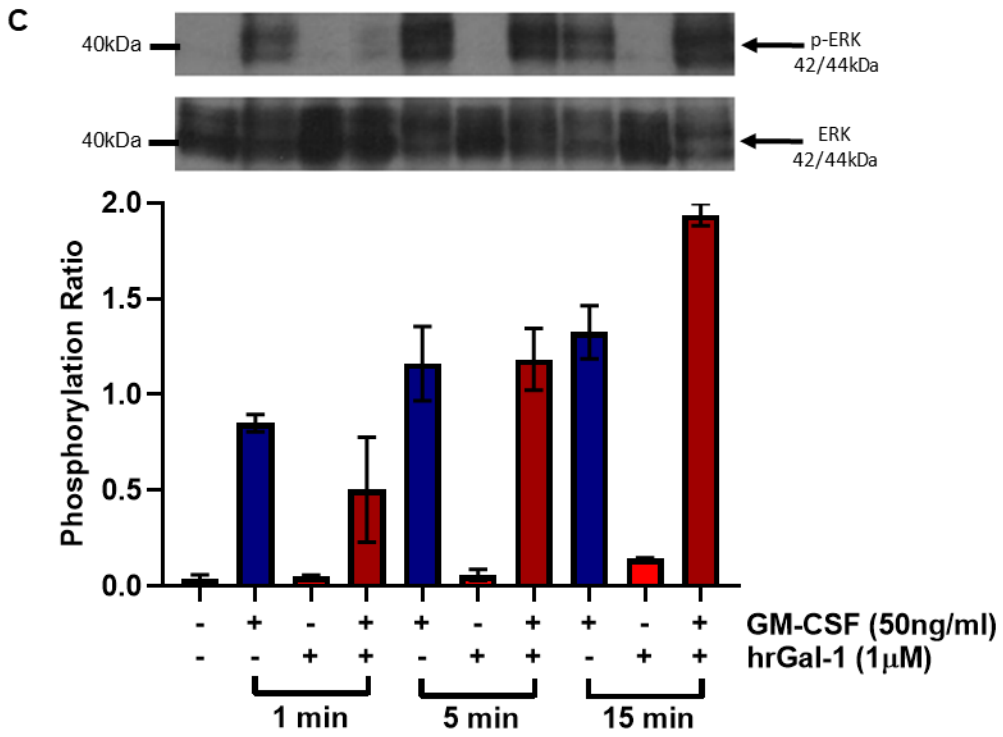



**Figure 51. Gal-1 delayed GM-CSF induced phosphorylation of signalling pathways.**
Neutrophils isolated from human peripheral blood were incubated (1min, 5min and 15mins) in RPMI Medium ± GM-CSF (50ng/ml) ± hrGal-1 (1μM). Following stimulation cells were lysed in hot loading buffer, sheared with a syringe and blotted for phosphorylation of AKT (**A**), P38 (**B**) and ERK (**C**). Representative blots are inlaid. Densitometry analysis was performed to quantify protein in bands. Results are shown as a ratio of phosphorylated protein to total. Cumulative data is shown as the mean ± SEM. n=2.

### *3.7.5. Early Phosphatidylserine Exposure*

AnxV binding was used to detect initial PS exposure on neutrophils following 1h treatment with hrGal-1 (1µM). Results shown in figure 52, revealed that compared to control neutrophils, hrGal-1 (1h) caused significantly increased PS exposure on the neutrophil surface (22.30% ± 6.78% vs 36.17% ± 4.62%, p = 0. 0317).

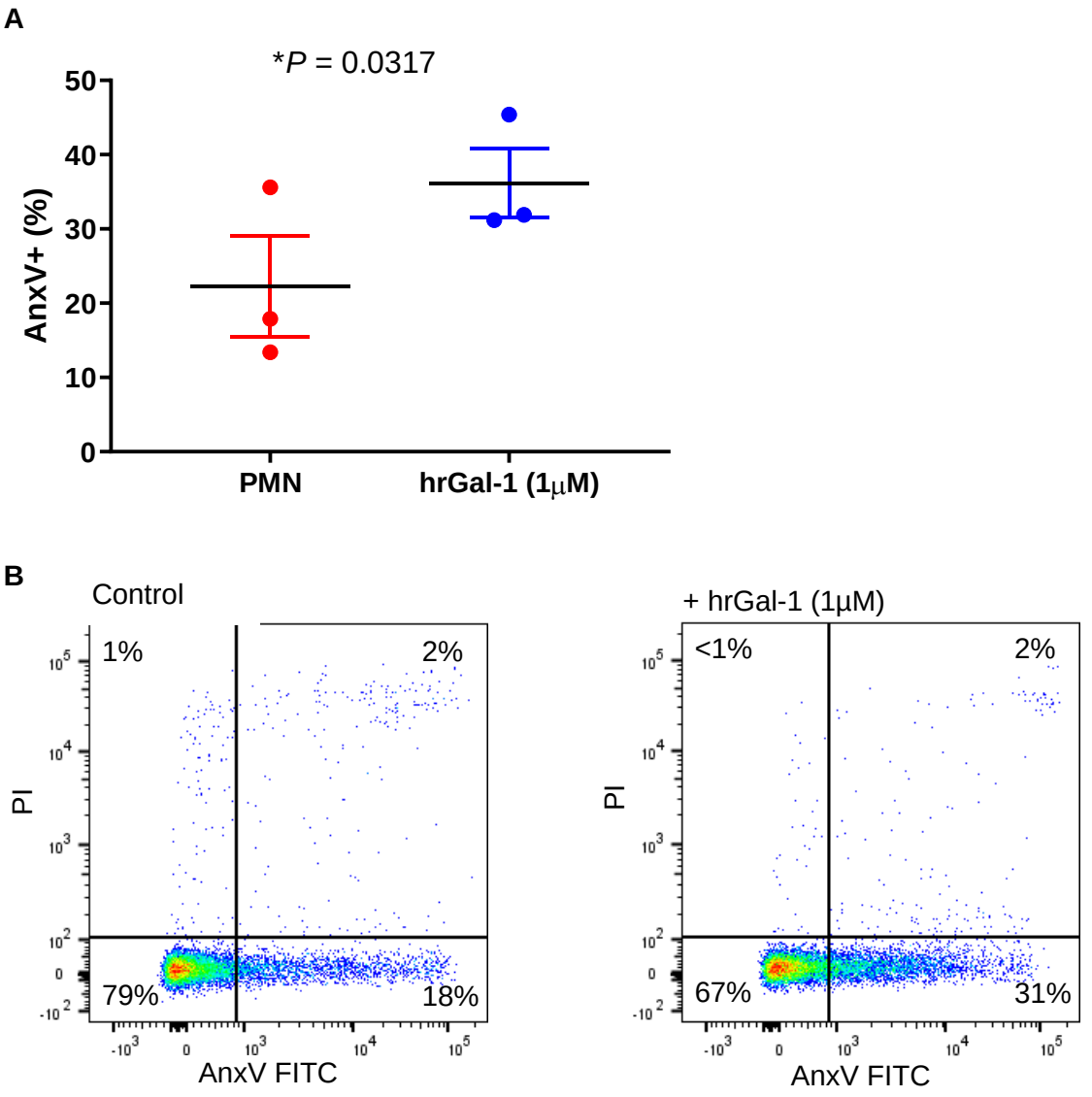
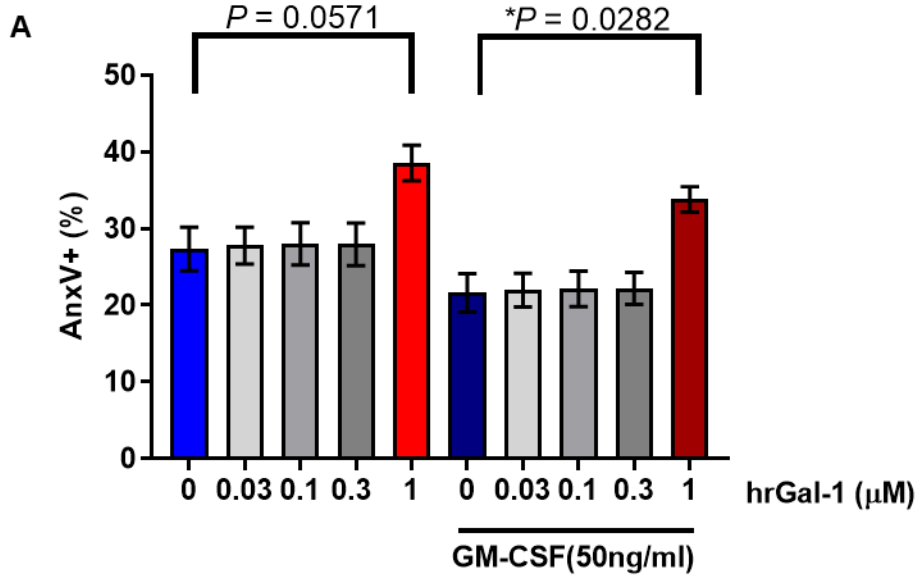



**Figure 52. Gal-1 increases early phosphatidylserine exposure on the surface of neutrophils.**
Neutrophils isolated from human peripheral blood were suspended in RPMI + 5% autologous serum, hrGal-1 (1µM) was added to cells for 1h incubation and PS exposure was detected by AnxV staining and analysed by flow cytometry. Cumulative data for percentage of neutrophils AnxV+ is shown as the mean ± SEM (**A**) with representative flow plots for each treatment (**B**). Statistical analysis was performed using a paired t test and results are considered significant when P < 0.05. n=3.

Similarly, AnxV binding was used to detect initial PS exposure on neutrophils following 4h (0.03, 0.1, 0.3 and 1μM) hrGal-1 ± hrGM-CSF (50ng/ml). After 4h neutrophils treated with hrGal-1 (1μM) still maintained increased PS exposure compared to unstimulated cells, this was not modified further by co-incubation with GM-CSF. As shown in figure 53 neutrophils incubated with GM-CSF had significantly increased phosphatidylserine externalisation in response to hrGal-1 (1μM) (AnxV+ %; 21.62% ± 2.52% vs 33.82% ± 1.66%, p = 0.0282).


A
P = 0.0571
*P = 0.0282
AnxV+ (%)
50
40
30
20
10
0
0 0.03 0.1 0.3 1 0 0.03 0.1 0.3 1
hrGal-1 (μM)
GM-CSF(50ng/ml)

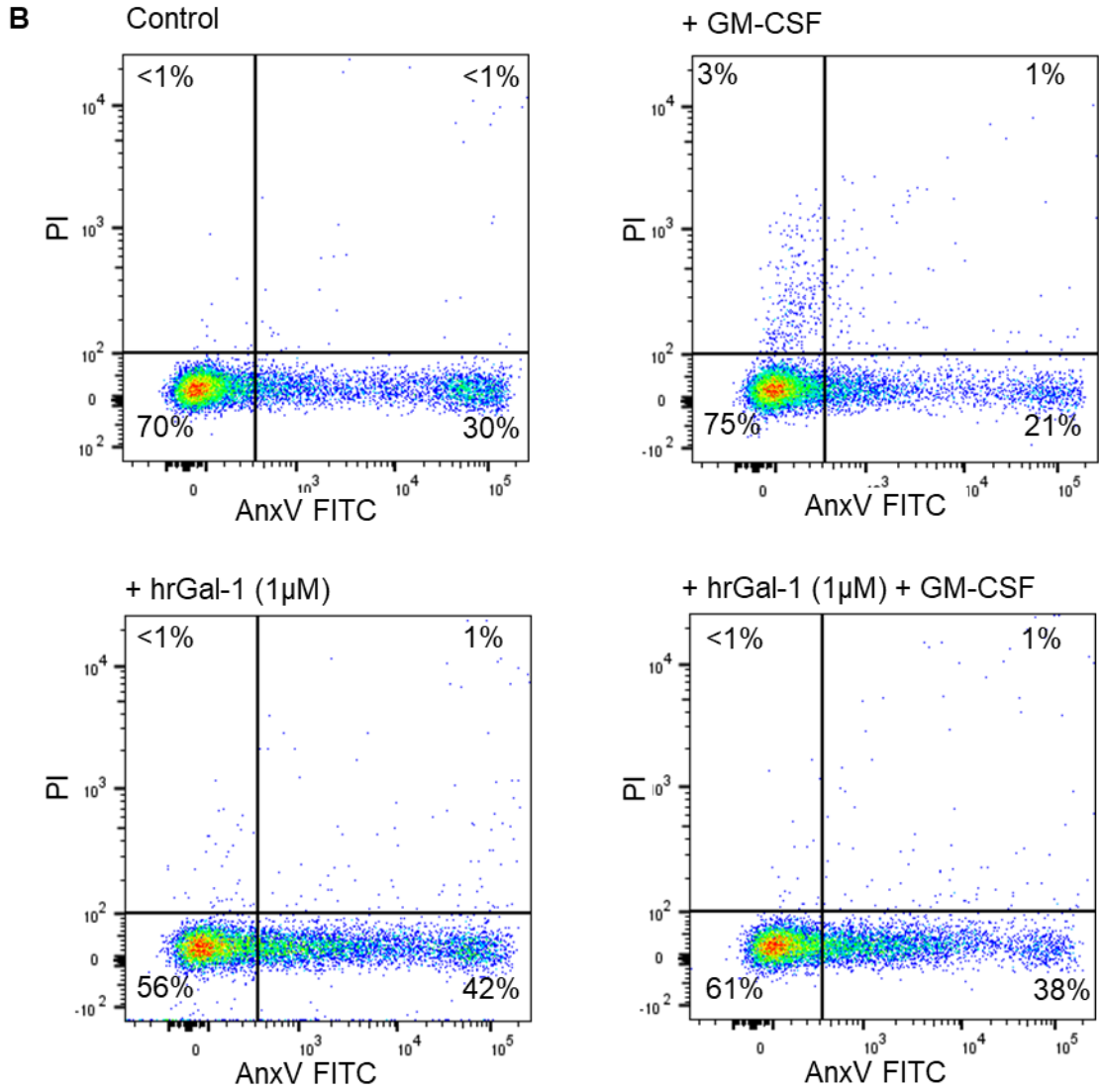

B
Control
<1%
<1%
70%
30%
PI
AnxV FITC
+ GM-CSF
3%
1%
75%
21%
+ hrGal-1 (1μM)
<1%
1%
56%
42%
+ hrGal-1 (1μM) + GM-CSF
<1%
1%
61%
38%

**Figure 53. Gal-1 maintains phosphatidylserine exposure on the surface of neutrophils.**
Neutrophils isolated from human peripheral blood were suspended in RPMI + 5% autologous serum. Cells were incubated for 4h ± GM-CSF (50ng/ml) ± hrGal-1 (0.03, 0.1, 0.3 or 1μM). Phosphatidylserine exposure was detected by AnxV staining and analysed by flow cytometry. Cumulative data for percentage of neutrophils AnxV+ is shown as the mean ± SEM (**A**) with representative flow plots for each treament (**B**). Statistical analysis was performed using a one-way ANOVA and results are considered significant when $P < 0.05$. n=5.

### *3.7.6. AnnexinA1 Expression*

Annexin A1 (AnxA1) is an endogenous protein that exerts anti-inflammatory and pro-resolving effects. Endogenous AnxA1 is rapidly translocated to the neutrophil surface upon activation and can override pro-survival signals to induce neutrophil apoptosis.

In this study surface and total AnxA1 levels were assessed on/in neutrophils at 0h and following incubations of 4h and 20h ± GM-CSF (50ng/ml) ± hrGal-1 (1µM). Results demonstrated no significant modulation in surface AnxA1 after 4h and 20h compared to levels expressed on freshly isolated cells. As displayed in figure 54A, stimulation with GM-CSF and/or hrGal-1 did not result in any notable modulation of surface AnxA1 compared to control cells.

Compared to surface expression, total AnxA1 levels were approximately 250-fold higher, indicating that the protein is predominantly intracellular. Total AnxA1 levels increased over time, with the highest expression of the protein detected at 20h (a time point where neutrophils are known to undergo spontaneous apoptosis). Results, shown in figure 54B, also revealed a trend towards reduced AnxA1 levels for neutrophils stimulated with GM-CSF, however this was not significant. Furthermore, data at 20h suggests that this reduction may be partially reversed by the co-incubation of cells with hrGal-1, whereas there was no notable effect revealed for hrGal-1 alone (similar to flow cytometry AnxV/PI data in section 3.3.1).

To assess AnxA1 release from neutrophils, cell free culture media was collected after 4h and 20h incubation and analysed by Western blot. Both the 4h and 20h time points showed the same trend, whereby GM-CSF markedly reduced neutrophil release of

AnxA1, as shown in figure 54C. Co-incubation with hrGal-1 reversed this effect, however the differences were less prominent at 4h compared to the 20h incubation. Notably, no AnxA1 was detected in the negative control which contained culture media with 5% autologous serum.

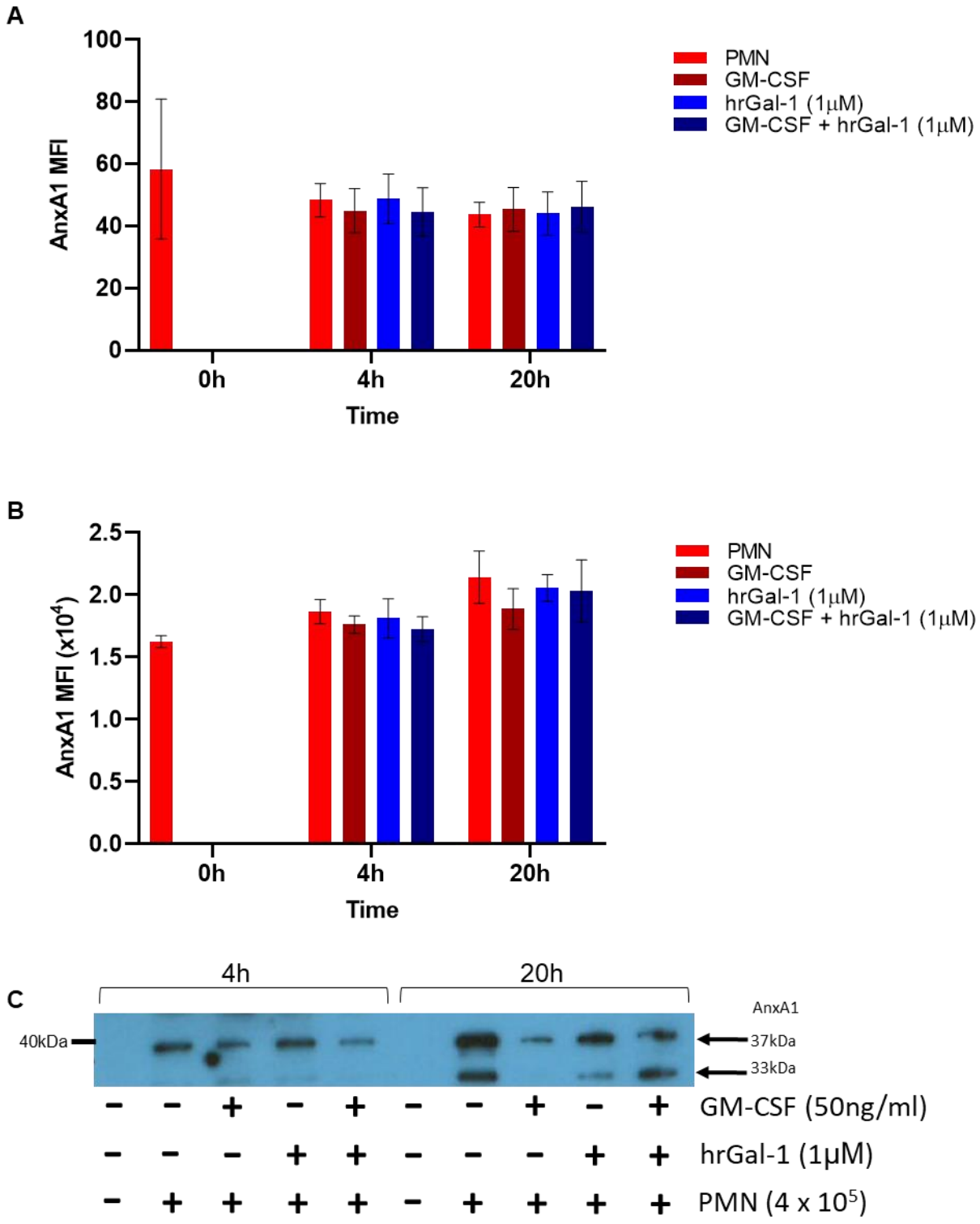


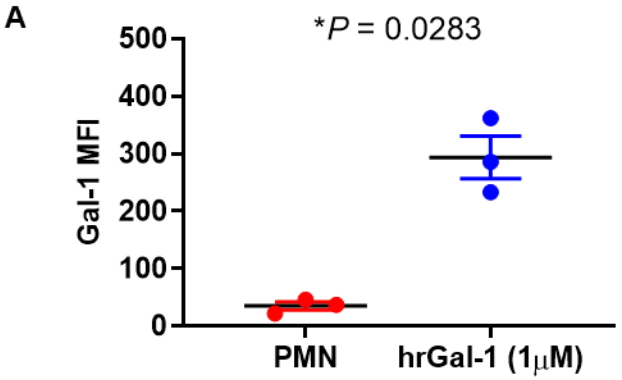

**Figure 54. Gal-1 reverses the effect of GM-CSF on AnxA1 release by apoptotic neutrophils.**
Neutrophils isolated from human peripheral blood were incubated in RPMI + 5% autologous serum ± hrGal-1 (1μM) ± GM-CSF (50ng/ml) for 4h and 20h. At 0h, 4h and 20h neutrophil surface (**A**) and total (**B**) AnxA1 was analysed by flow cytometry. Cell free culture media was assayed for AnxA1 by Western Blot (**C**), results shown for one representative donor (4h left hand side, 20h right hand side). Cumulative data are shown as the mean ± SEM. Statistical analysis was performed using a two-way ANOVA and in all cases results are considered significant when $P < 0.05$. n=3.

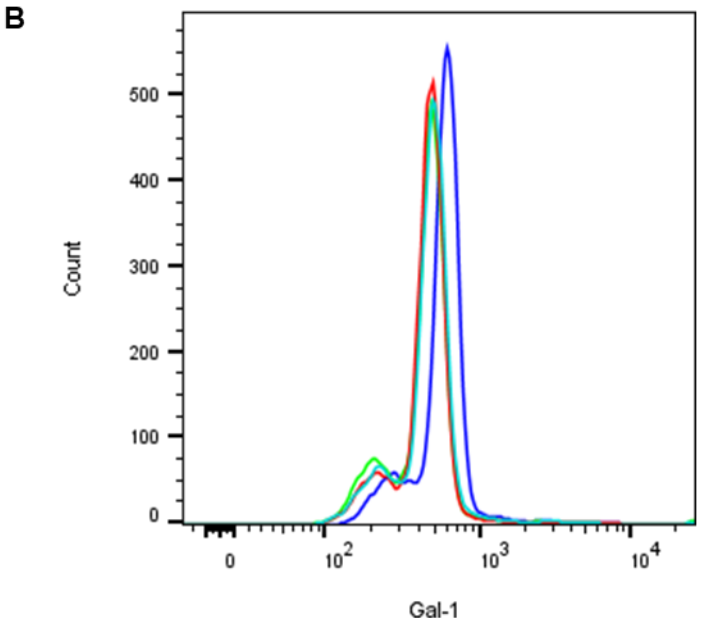
### *3.8. Gal-1 Expression and Binding on Human Neutrophils.*

The relative level of Gal-1 on the surface of neutrophils as they undergo apoptosis was assessed to determine if it is modulated with the apoptotic process. Furthermore, if the Gal-1 detected on the surface was coming from the addition of the exogenous protein binding to the cell or if it originated intracellularly and if its exposure at the cell surface was modulated as a consequence of undergoing apoptosis was also investigated. Finally, whether exogenous Gal-1 had a higher affinity for binding to viable or apoptotic neutrophils was also addressed. In these assays extracellular Gal-1, exogenously bound and/or endogenously expressed, was detected on neutrophils using flow cytometry.

#### *3.8.1. Viable Neutrophils*

Incubation of neutrophils with hrGal-1 (1μM) for 1h confirmed that Gal-1 binds to viable neutrophils, as indicated by significantly increased Gal-1 detected on the surface of neutrophils incubated with the exogenous protein ($p = 0.0283$, figure 55A). Inclusion of lactose (30mM), which is known to inhibit galectin binding, notably reduced surface Gal-1 (as shown in figure 55B), and therefore further verified that the initial increase was the result of exogenous protein binding to the neutrophil surface in a carbohydrate-dependent manner.



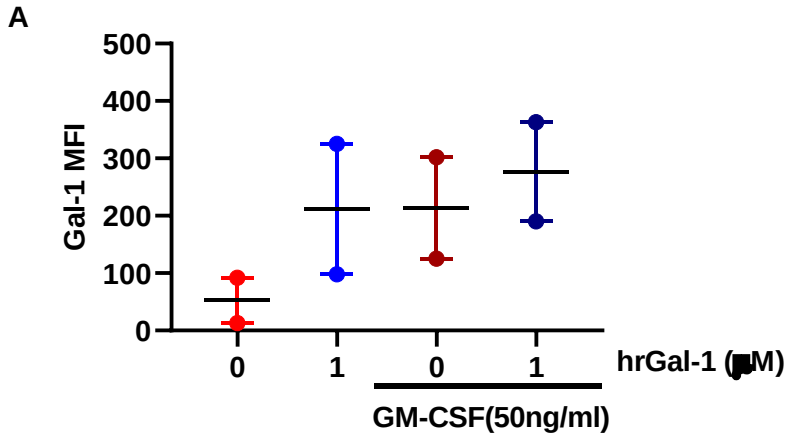




| 1h Incubation | Gal-1 MFI |
|---|---|
| PMN | 16 |
| hrGal-1 (1μM) | 140 |
| Lactose (30mM) | 15 |
| hrGal-1 (1μM) + Lactose (30mM) | 31 |

**Figure 55. Gal-1 binds to the surface of viable neutrophils.**
Neutrophils isolated from human peripheral blood were suspended in RPMI + 5% autologous serum for 1h ± hrGal-1 (1μM) (**A**) and co-incubated with lactose (30mM) (**B**). Surface Gal-1 levels were analysed by flow cytometry and cumulative data is shown as the mean ± SEM. Histogram and Gal-1 median fluorescence intensity values shown for a representative donor. Statistical analysis was performed using a paired t test with results considered significant when $P < 0.05$. n=3.

### *3.8.2. Exogenous Gal-1 during Neutrophil Apoptosis*

Neutrophils incubated for 20h in 5% autologous serum had similar levels of Gal-1 on their surface compared to viable neutrophils and therefore demonstrated that Gal-1 is not increased on the surface of unstimulated apoptotic neutrophils in comparison to viable cells, as shown in figure 56A. The addition of GM-CSF (50ng/ml) and/or hrGal-1 (1μM) to the overnight incubation resulted in increased Gal-1 detected on the neutrophil surface, however this trend was not significant. Incubation with lactose (30mM) did not have any effect on the already bound/expressed levels of Gal-1 on apoptotic neutrophils (figure 56B) and therefore indicated that Gal-1 is not easily dissociated from the neutrophil cell surface.



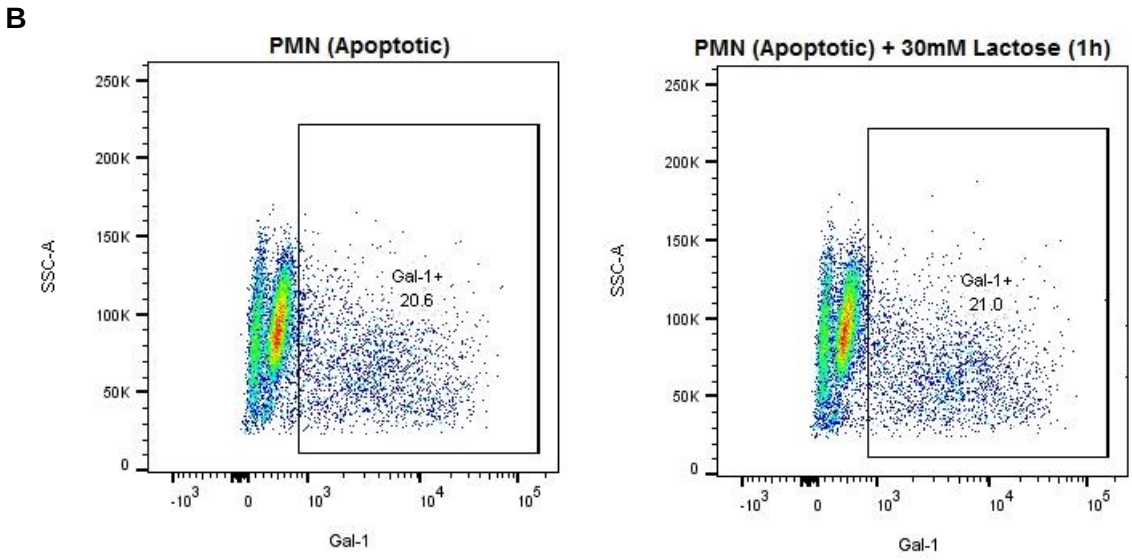




**Figure 56. Gal-1 on the surface of apoptotic neutrophils is not removed by lactose.**
Neutrophils isolated from human peripheral blood and incubated in RPMI 5% autologous serum ± GM-CSF (50ng/ml) ± hrGal-1 (1μM) for 20h (**A**) and following 20h incubation, lactose (30mM) was added to apoptotic neutrophils for 1h (**B**). Surface Gal-1 levels were analysed by flow cytometry and cumulative data is shown as the mean ± SEM. Flow cytometry plots are shown for a representative donor. n=2.

### *3.8.3. Apoptotic Neutrophils*

Incubation of apoptotic neutrophils with hrGal-1 (1μM) for 1h resulted in an increase in Gal-1 on the surface of the neutrophil population, as shown in figure 57A. When the whole neutrophil population was plotted as a histogram for Gal-1 MFI two distinct peaks were revealed, as in figure 57B. Both control neutrophils and hrGal-1 treated cells had a high MFI population that was comparably equal in height and distribution and did not shift in response to hrGal-1. Conversely the population which formed a peak with lower Gal-1 expression was shown to increase in levels of the protein in response to incubation with hrGal-1. On further assessment the population with high Gal-1 expression could be classified as late apoptotic based on the decreased forward and side scatter (indicative of cell shrinkage - a classic phenotype of apoptotic cells). The population with lower Gal-1 expression had a higher forward and side scatter when plotted and formed a tighter cloud of cells, typical of live neutrophils, as shown in plots in figure 57C. This data indicates that hrGal-1 does not bind to the surface of neutrophils undergoing the latter stages of apoptosis and thus committed to cell death. Furthermore, in this assay hrGal-1 maintains the ability to bind living neutrophils in the presence of apoptotic cells and any mediators they have released whilst undergoing cell death.

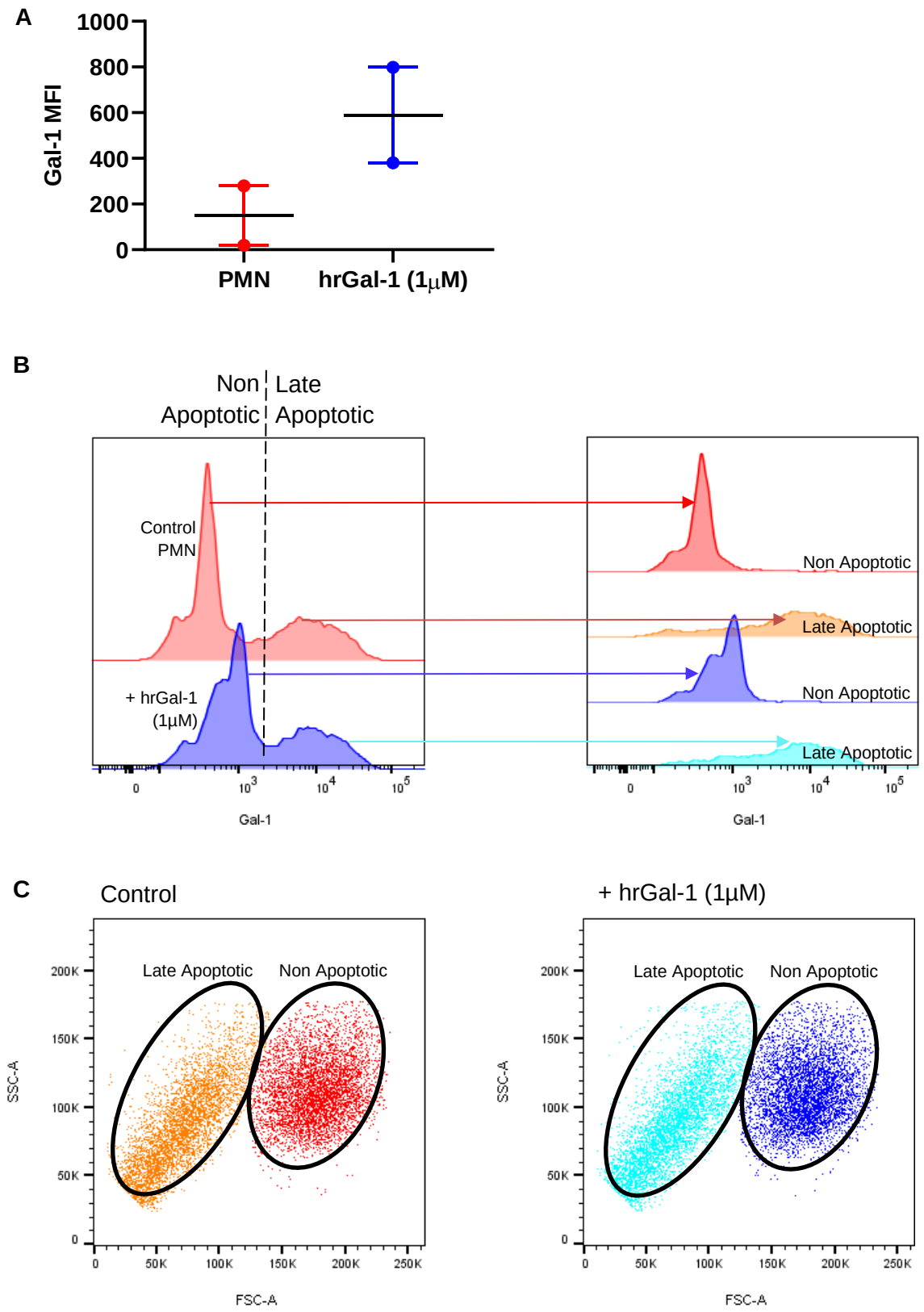



**Figure 57. Gal-1 does not bind to the surface of late apoptotic neutrophils.**
Neutrophils isolated from human peripheral blood were incubated in RPMI + 5% autologous serum for 20h. Following incubation hrGal-1 (1μM) was added to apoptotic neutrophils for 1h. Surface Gal-1 levels were analysed by flow cytometry and cumulative data is shown as the mean ± SEM (**A**). Histograms display Gal-1 MFI (left) and further indicate non apoptotic and late apoptotic cell populations (right) for a representative donor (**B**) also presented as FSC-A vs SSC-A flow cytometry plots (**C**). n=2.

### *3.9. Gal-1 Receptor Expression and Binding on Human Neutrophils.*

Assessment of galectin receptor expression on neutrophils was performed ± GM-CSF (50ng/ml). GM-CSF was not shown to modulate levels of the transmembrane glycoproteins CD43 (figure 58A) or CD44 (figure 58B). In this assay neutrophils were also incubated ± hrGal-1 (0.03μM, 0.1μM, 0.3μM and 1μM) in addition to GM-CSF. As shown in figures 58A and B there was no notable modulation to the capability of either the CD43 or CD44 antibodies to bind to the cell surface as a result of the addition of hrGal-1. In contrast, CD45 was markedly upregulated on the neutrophil surface in response to 4h incubation with the pro-survival cytokine, as shown in figure 58C. In addition, detection of CD45 using an anti-CD45 antibody was compromised by hrGal-1, with a concentration dependent response seen, whereby an increase in hrGal-1 resulted in the inverse level of anti-CD45 binding to the neutrophil. This was shown for neutrophils with and without GM-CSF (figure 58C) and suggested that hrGal-1 is binding to the CD45 on the cell surface and thus prohibiting antibody binding to the antigen.

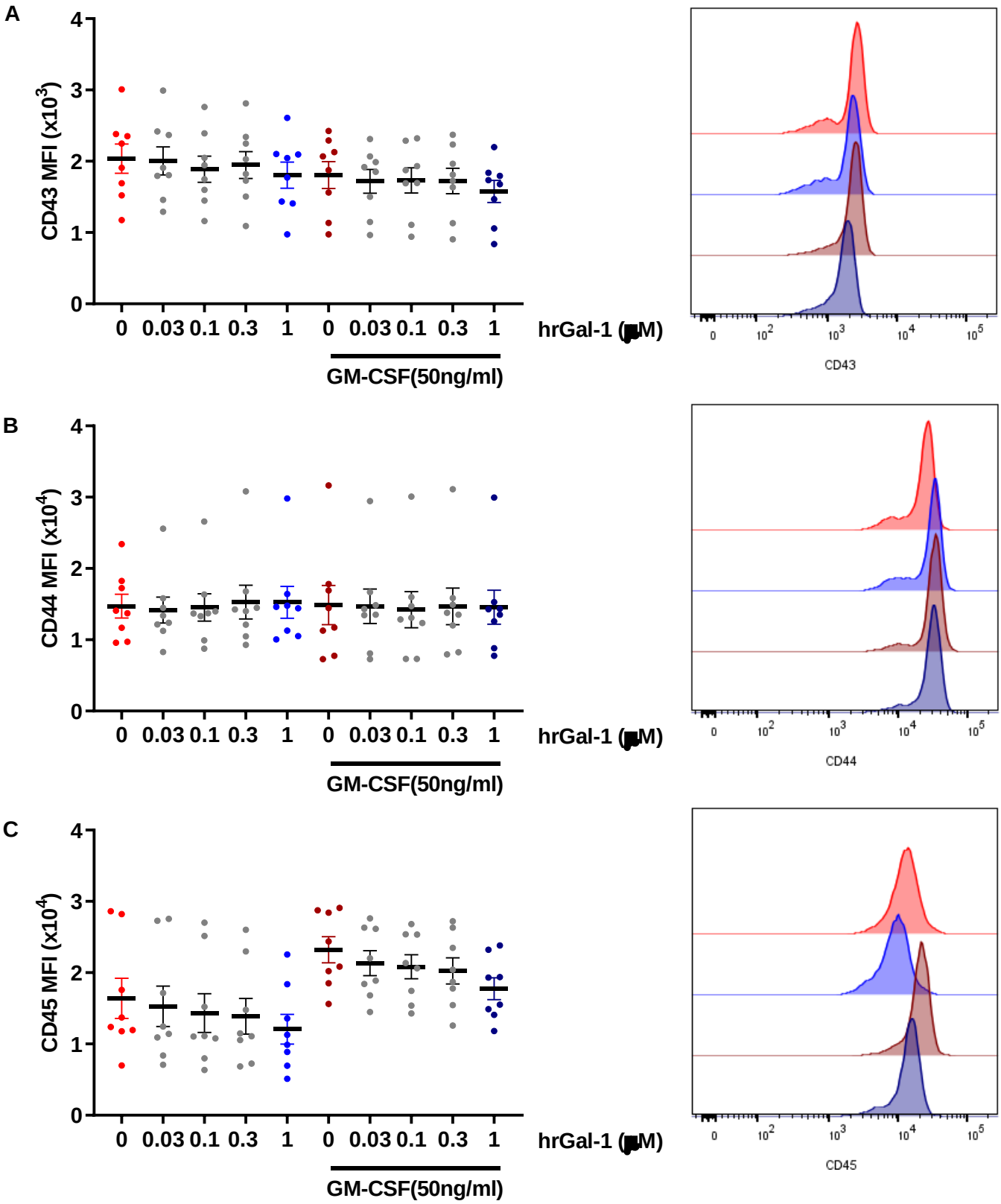

A
CD43 MFI (x10^3)
0 1 2 3 4
0 0.03 0.1 0.3 1 0 0.03 0.1 0.3 1
hrGal-1 (μM)
GM-CSF(50ng/ml)
CD43
B
CD44 MFI (x10^4)
0 1 2 3 4
0 0.03 0.1 0.3 1 0 0.03 0.1 0.3 1
hrGal-1 (μM)
GM-CSF(50ng/ml)
CD44
C
CD45 MFI (x10^4)
0 1 2 3 4
0 0.03 0.1 0.3 1 0 0.03 0.1 0.3 1
hrGal-1 (μM)
GM-CSF(50ng/ml)
CD45

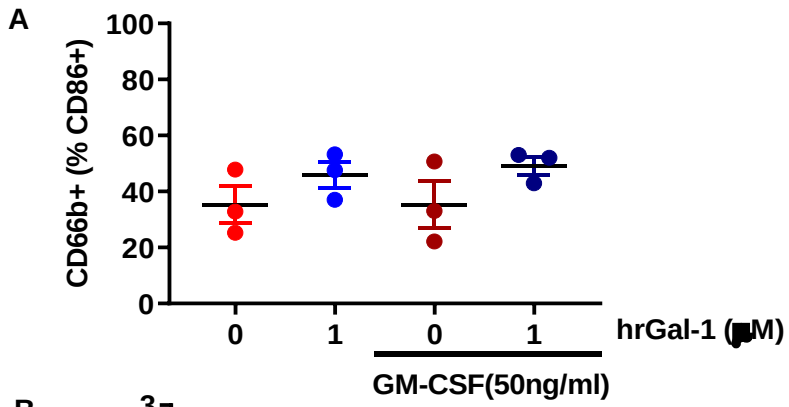
**Figure 58. The Gal-1 receptor CD45 was upregulated on neutrophils in response to GM-CSF.**
Neutrophils isolated from human peripheral were incubated (4h) in RPMI + 5% autologous serum ± GM-CSF (50ng/ml) ± hrGal-1 (0.03, 0.1, 0.3 or 1μM). Expression of galectin receptors on the neutrophil surface were assessed by CD43 (**A**), CD44 (**B**) and CD45 (**C**) antibody binding and analysed by flow cytometry. The median fluorescence intensity (MFI) for each receptor was determined from the single cell population of neutrophils. Cumulative data are shown as the mean ± SEM (left) with representative histograms (right). Statistical analysis was performed using a one-way ANOVA and in all cases results are considered significant when $P < 0.05$. n=8.

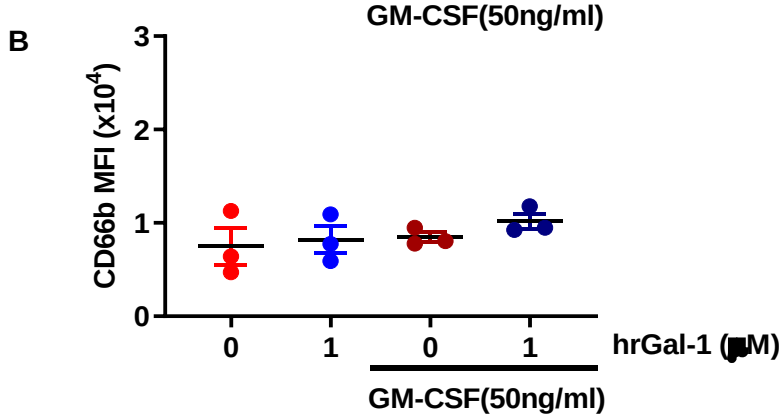
### *3.10. The Effect of exogenous Gal-1 on Efferocytosis.*

Efferocytosis assays were performed to determine whether neutrophils undergoing apoptosis ± hrGal-1 (1μM) ± GM-CSF (50ng/ml) would be efferocytosed any differently as a result of either agent. Furthermore, the addition of hrGal-1 (1μM) to both viable and apoptotic neutrophils (1h) prior to and during their addition to macrophages was used to determine whether Gal-1 served as an opsonin. Efferocytosis was assessed by overlaying apoptotic human neutrophils on human macrophages for 1h and then analysing uptake by flow cytometry. For consideration of macrophage efferocytic satiation two different macrophages to apoptotic neutrophils ratios were assessed, by overlaying $2x10^6$ or $5x10^6$ neutrophils, yielding a ratio of 1:2 or 1:5 respectively.

#### *3.10.1. Exogenous Gal-1 during Neutrophil Apoptosis*

After 1h approximately 35% of macrophages had ingested an apoptotic neutrophil when they were plated at a ratio of 1:2. Results indicated that there was no difference in phagocytic uptake of neutrophils by macrophages as a result of cells undergoing apoptosis in the presence ± hrGal-1 (1μM) ± GM-CSF (50ng/ml). The highest MFI values were observed with the co-treatment (figure 59B). As expected, when the ratio of apoptotic PMN to macrophages was increased, this resulted in an increased percentage of macrophages having ingested neutrophils (approximately 10-20% more macrophages) (figure 60A). When the baseline level of efferocytosis was increased the differences with Gal-1 treatment became less obvious, although the highest percentage of efferocytosis was observed with GM-CSF plus hrGal-1. Again, the highest CD66b MFI levels were following co-treatment (figure 60B).


A
CD66b+ (% CD86+)
100
80
60
40
20
0
0
1
0
1
hrGal-1 (μM)
GM-CSF(50ng/ml)


B
CD66b MFI (x10^4)
3
2
1
0
0
1
0
1
hrGal-1 (μM)
GM-CSF(50ng/ml)

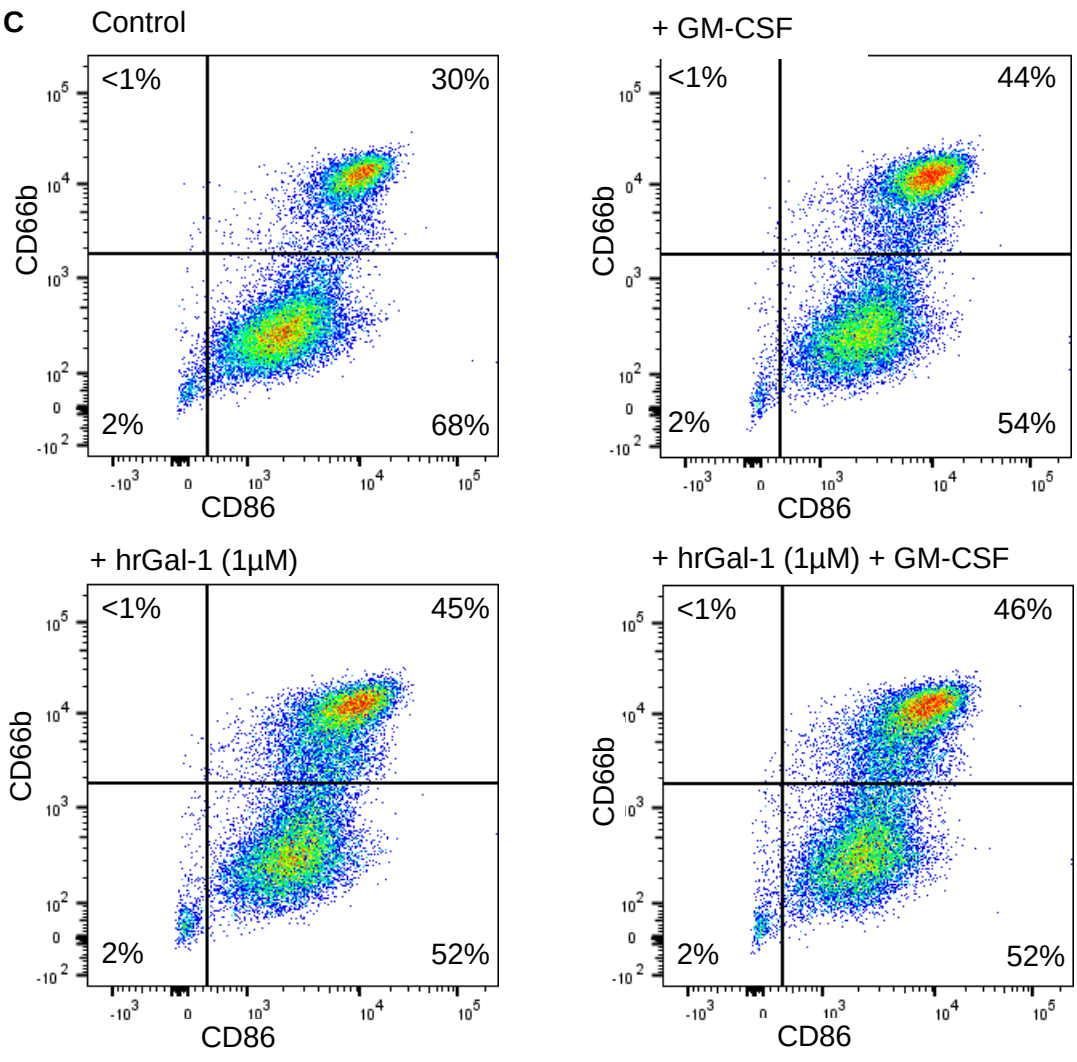
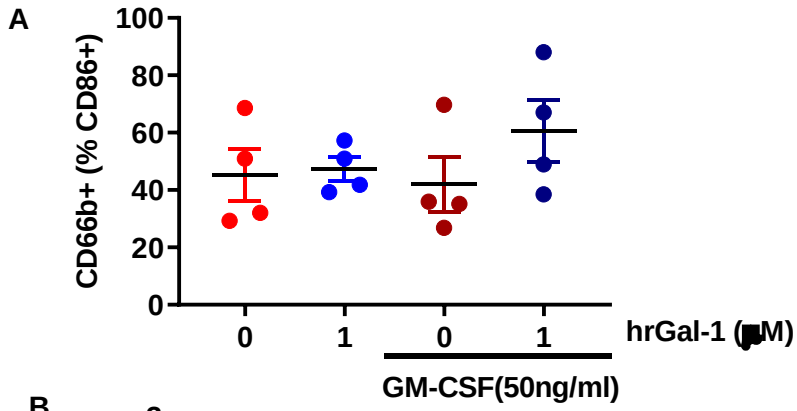
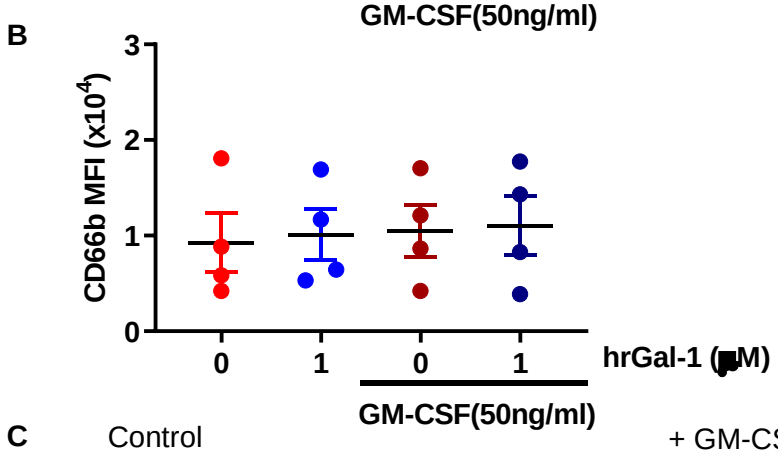

C
Control
<1%
30%
2%
68%
+ GM-CSF
<1%
44%
2%
54%
+ hrGal-1 (1μM)
<1%
45%
2%
52%
+ hrGal-1 (1μM) + GM-CSF
<1%
46%
2%
52%
CD66b
CD86

**Figure 59. Gal-1 addition to neutrophils undergoing apoptosis did not affect macrophage efferocytosis or satiation (I).**
Neutrophils were incubated (20h) in RPMI + 5% autologous serum ± GM-CSF (50ng/ml) ± hrGal-1 (1μM) to undergo apoptosis. Efferocytosis was assessed by overlaying apoptotic neutrophils (2 x $10^6$) on macrophages (1 x $10^6$) for 1h. Remaining neutrophils were washed away, and macrophages were collected. Cells were permeabilised, stained with an antibody against a human macrophage-specific marker (CD86) and a human neutrophil specific marker (CD66b) and analysed by flow cytometry. The macrophage population was assessed for percentage CD66b+ (**A**) and the positive population assessed for CD66b fluorescence intensity and displayed as the median values (**B**) with representative plots for quadrant gating displayed (**C**). Statistical analysis was performed using a one-way ANOVA with results considered significant if $P < 0.05$. n=3.


A
CD66b+ (% CD86+)
100
80
60
40
20
0
0
1
0
1
hrGal-1 (μM)
GM-CSF(50ng/ml)


B
CD66b MFI (x10^4)
3
2
1
0
0
1
0
1
hrGal-1 (μM)
GM-CSF(50ng/ml)

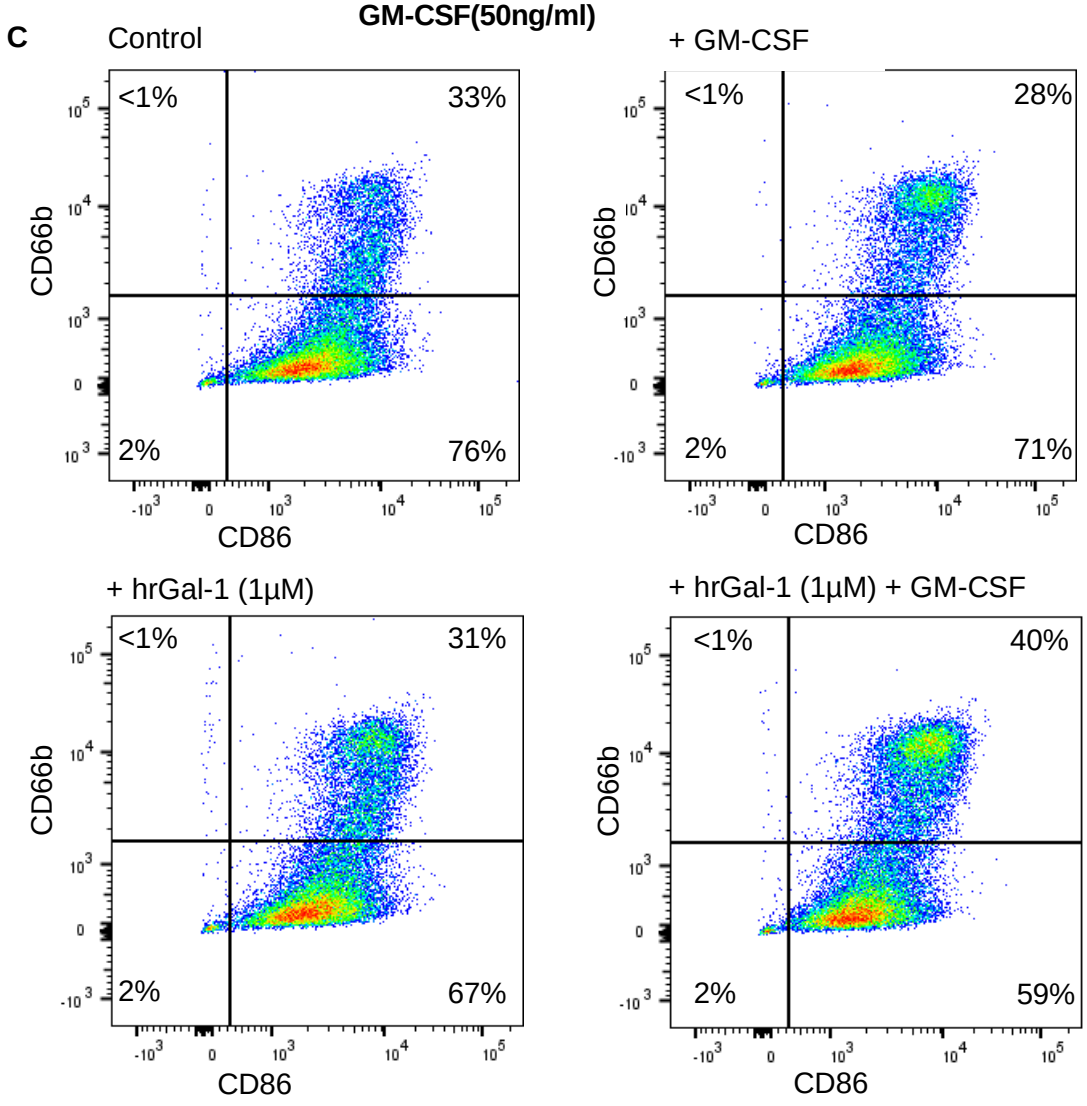

C
Control
<1%
33%
2%
76%
+ GM-CSF
<1%
28%
2%
71%
+ hrGal-1 (1μM)
<1%
31%
2%
67%
+ hrGal-1 (1μM) + GM-CSF
<1%
40%
2%
59%
CD66b
CD86

**Figure 60. Gal-1 addition to neutrophils undergoing apoptosis did not affect macrophage efferocytosis or satiation (II).**
Neutrophils were incubated (20h) in RPMI + 5% autologous serum ± GM-CSF (50ng/ml) ± hrGal-1 (1μM) to undergo apoptosis. Efferocytosis was assessed by overlaying apoptotic neutrophils (5 x $10^6$) on macrophages (1 x $10^6$) for 1h. Remaining neutrophils were washed away, and macrophages were collected. Cells were permeabilised, stained with an antibody against a human macrophage-specific marker (CD86) and a human neutrophil specific marker (CD66b) and analysed by flow cytometry. The macrophage population was assessed for percentage CD66b+ (**A**) and the positive population assessed for CD66b fluorescence intensity and displayed as the median values (**B**) with representative plots for quadrant gating displayed (**C**). Statistical analysis was performed using a one-way ANOVA with results considered significant if $P < 0.05$. n=4.

### *3.10.2. Exogenous Gal-1 as an Opsonin*

The addition of hrGal-1 (1μM) to both viable and apoptotic neutrophils (1h) prior to and during their incubation with macrophages was used to determine whether Gal-1 served as an opsonin. Here, efferocytosis of neutrophils was not modulated by hrGal-1 being added to apoptotic cells for 1h prior to and during their overlay on macrophages. These results showed that neither the percentage of macrophages containing neutrophils (figure 61A) or the relative number of cells ingested (figure 61B) was affected by addition of hrGal-1. Likewise, viable neutrophils treated with hrGal-1 before and during their co-incubation with macrophages revealed no significant effect on either macrophage percentage or MFI (figures 62A & B respectively) in response to the inclusion of hrGal-1 in the assay. These results suggest that Gal-1 does not act as an opsonin for efferocytosis.

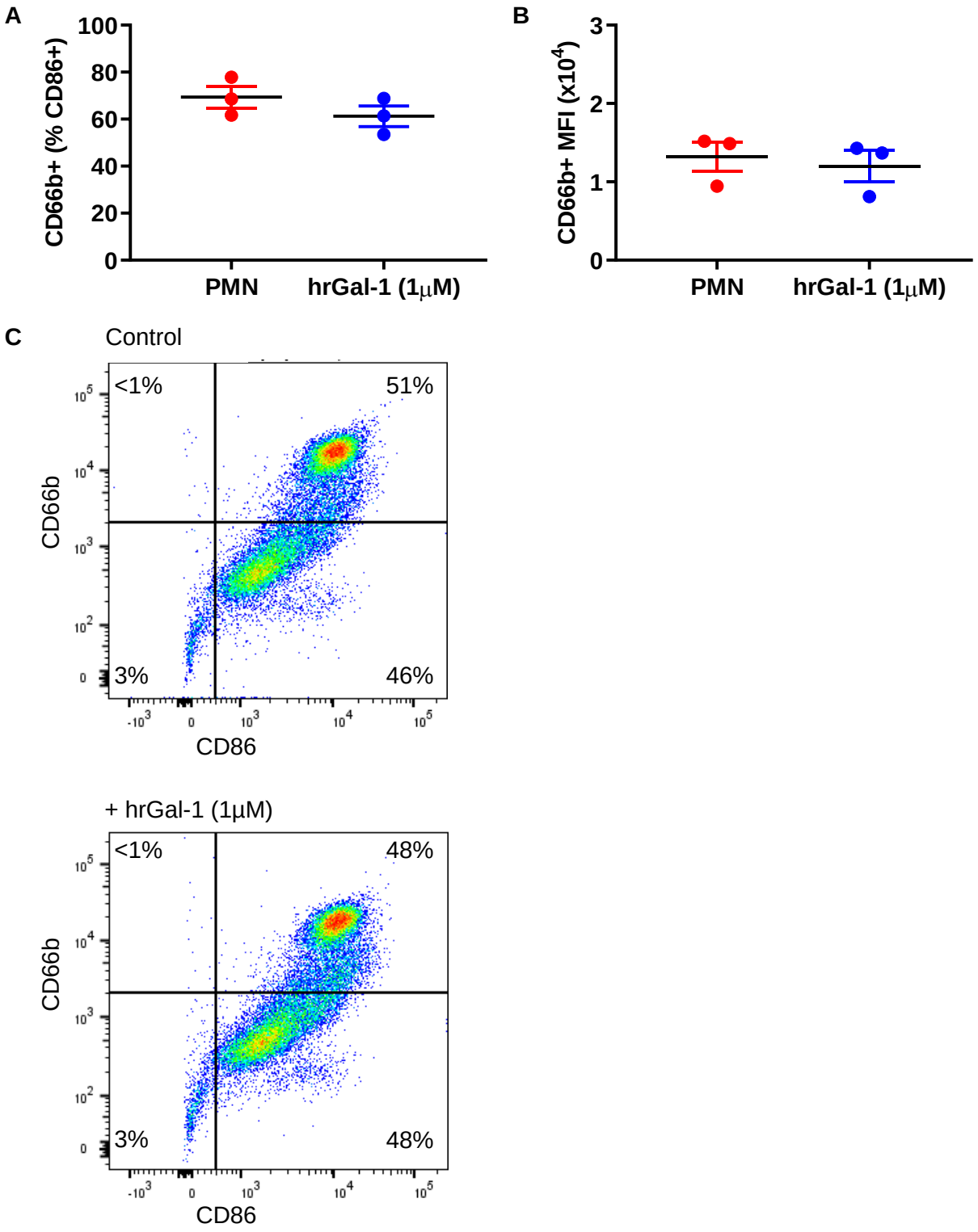



**Figure 61. Gal-1 does not act as an opsonin for macrophage efferocytosis of apoptotic neutrophils.** Efferocytosis was assessed by overlaying apoptotic neutrophils (2 x $10^6$) on macrophage (1 x $10^6$) for 1h ± hrGal-1 (1μM). For apoptotic cells neutrophils were incubated in RPMI – 1640 Medium + L-glut + 5% autologous serum for 20h, hrGal-1 was then added to apoptotic neutrophils 1h prior to overlaying on macrophage and maintained within the media during co-incubation. Remaining neutrophils were then washed away and macrophage collected. Cells were permeabilised and stained with antibodies against human macrophage (CD86) and human neutrophils (CD66b). The macrophage population was analysed by flow cytometry for percentage CD66b+ (**A**) and the positive population assessed for CD66b fluorescence intensity and displayed as the median values (**B**) with representative plots for quadrant gating displayed (**C**). Statistical analysis was performed using a paired t test with results considered significant if $P < 0.05$, n=3.

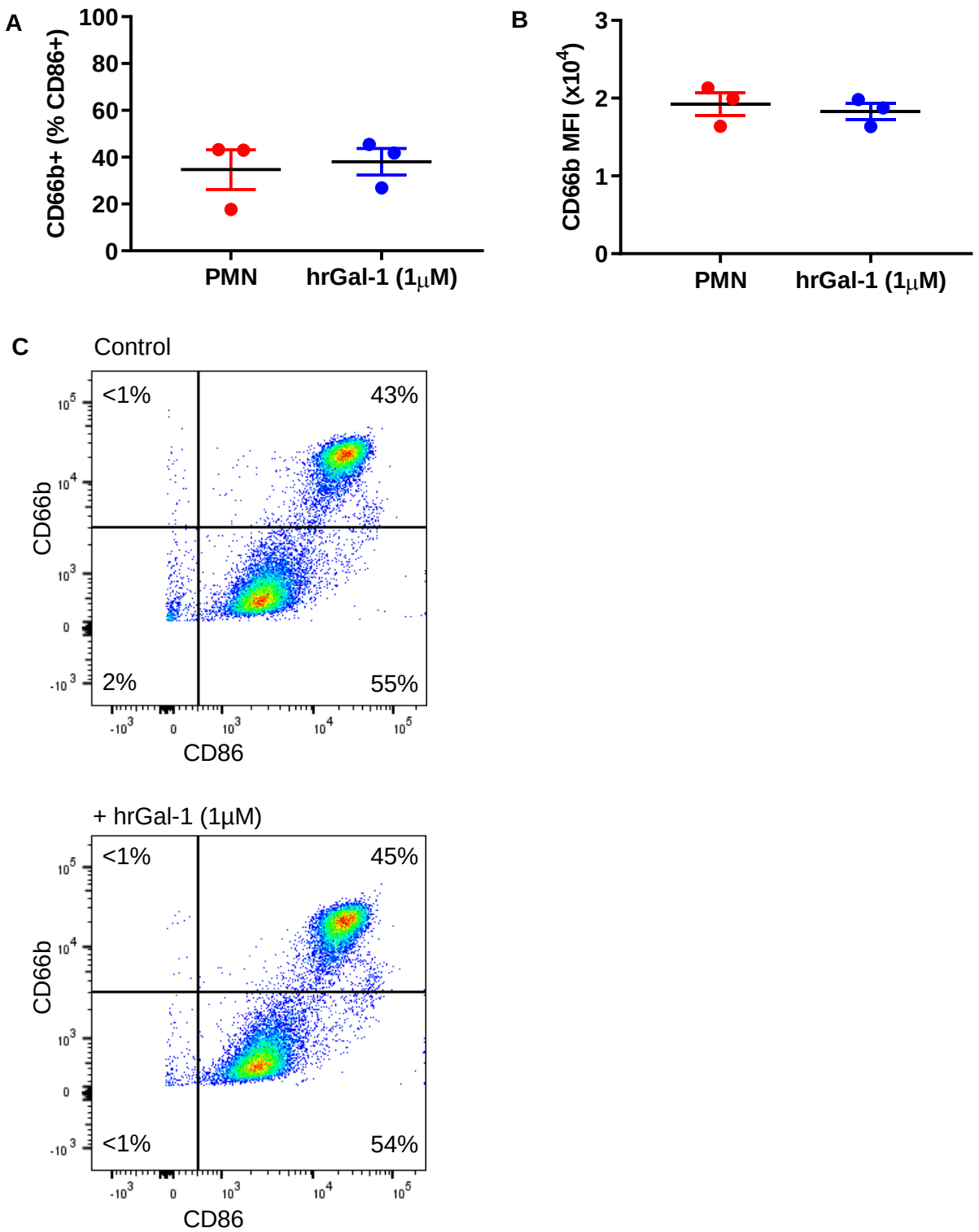



**Figure 62. Gal-1 does not act as an opsonin for macrophage efferocytosis of viable neutrophils.**
Efferocytosis was assessed by overlaying viable neutrophils (2 x $10^6$) on macrophage (1 x $10^6$) for 1h ± hrGal-1 (1μM). HrGal-1 was added to neutrophils 1h prior to overlaying on macrophage and maintained within the media during co-incubation. Remaining neutrophils were then washed away and macrophage collected. Cells were permeabilised and stained with antibodies against human macrophage (CD86) and human neutrophils (CD66b). The macrophage population was analysed by flow cytometry for percentage CD66b+ (**A**) and the positive population assessed for CD66b fluorescence intensity and displayed as the median values (**B**) with representative plots for quadrant gating displayed (**C**). Statistical analysis was performed using a paired t test with results considered significant if $P < 0.05$, n=3.

## Chapter 4: Discussion

Considered to be predominantly endowed with anti-inflammatory actions Gal-1 targets various stages of the immune response. Its effects are varied depending on the target cell type and whether the actions are intracellular or extracellular. The promiscuous binding of Gal-1 also increases the complexity for defining the mechanisms of action; known receptors include CD43 and CD45 on T cells (Nguyen *et al.*, 2001; Hernandez *et al.*, 2006). Particularly pertinent to this project are its effects on leukocyte trafficking and apoptosis. Inhibition of T cell trafficking under both physiological and pathophysiological states, has been shown to be significantly inhibited in response to Gal-1 (Norling *et al.*, 2008). The ability of Gal-1 to induce the apoptosis of Th1 cells, as well as switch the immune response towards a Th2 phenotype, is thought to be central to its immunomodulatory functions (Rabinovich, Daly, *et al.*, 1999). In this regard, the protective effects of Gal-1 have been evidenced in several models of auto-immune disease, such as collagen-induced arthritis (Rabinovich, Daly, *et al.*, 1999; Iqbal *et al.*, 2013) and experimental autoimmune encephalomyelitis (Offner *et al.*, 1990), with the former providing the first link between Gal-1 and RA .

With regards to the innate immune system, administration of recombinant Gal-1 has been shown to reduce leukocyte trafficking *in vivo* (La *et al.*, 2003) and to inhibit neutrophil-endothelial interactions *in vitro* (Cooper *et al.*, 2008), whilst trafficking is enhanced in Gal-1 KO mice (Cooper *et al.*, 2008). A suggested role in resolution of inflammation stemmed from its presence in resolving exudates (Chiang *et al.*, 2008). Evidence for a role in neutrophil apoptosis and clearance is scant, with current literature suggesting that it may facilitate neutrophil clearance via a process termed

“preaperesis” whereby neutrophils are efferocytosed without undergoing apoptosis, but this has still to be definitively proven (Dias-Baruffi *et al.*, 2003; Stowell *et al.*, 2007, 2009). Specifically, Gal-1 has been shown to induce the outer membrane exposure of the common death signal phosphatidylserine in neutrophils independently of apoptosis (Stowell *et al.*, 2009). However findings from one study determined this response to be dependent on neutrophil phenotype, indicating that the phenomenon of PS exposure in the absence of apoptosis only occurs when cells are activated and the same effect is not seen on resting cells (Stowell *et al.*, 2007). Furthermore, it was also shown to be reversible as phosphatidylserine exposure reverts inwards again after Gal-1 removal (Stowell *et al.*, 2009), without altering cell viability.

These studies point towards a relationship between Gal-1 and neutrophil behaviour, particularly with respect to cell trafficking and viability, which has the potential to be fundamentally relevant to neutrophil-driven pathologies. Rheumatoid Arthritis provides an example where perpetuation of disease results from the recurrent movement of neutrophils from the blood to the targeted site, in this case the arthritic joint. The continual influx of inflammatory stimulated neutrophils in combination with the enhanced lifespan afforded to these cells crucially supplement the progression of inflammation and causation of chronic disease. Yet the impact of Gal-1 on neutrophil-driven pathologies is not well studied and remains poorly understood. Likewise, the roles of Gal-1 in the resolution of inflammation remains to be fully elucidated as well as whether the abilities of the protein to inhibit neutrophil recruitment and potentiate neutrophil clearance are responsible for mediating the anti-inflammatory properties demonstrated by Gal-1. Therefore, these aspects have been chosen for further

investigation and study by comprehensive examination of the role of Gal-1 on neutrophil recruitment and clearance in the arthritic joint.

## *4.1. Gal-1 Modulates Neutrophil Numbers in a Model of Acute Inflammation.*

### *4.1.1. Neutrophil Recruitment is Enhanced in the Absence of Gal-1.*

Significantly more neutrophils trafficked to the peritoneal cavity of Gal-1 null mice, compared to their WT counterparts during acute low dose zymosan (6h time point), indicating that in acute inflammation, neutrophil trafficking is increased in the absence of Gal-1. This result is in line with findings in the literature, with enhanced neutrophil trafficking in the colon of Gal-1 KO mice in response to infection with *C. rodentium* (Curciarello *et al.*, 2014). Leukocyte trafficking is also enhanced in the inflamed cremasters of Gal-1 KO mice in response to IL-1β (Cooper *et al.*, 2008).

It is well established that endogenous Gal-1 is expressed by endothelial cells and that this expression, as well as distribution, can be upregulated on the surface membrane by inflammatory cytokines (Baum, Pang, *et al.*, 1995; Baum, Seilhamer, *et al.*, 1995). It is therefore plausible that endogenous Gal-1 is produced by the endothelium to counter regulate neutrophil trafficking. More specifically that upregulation of Gal-1 on the endothelial cell surface occurs in response to stimulation by inflammatory cytokines as a mechanism of impeding recruitment and preventing an overzealous infiltration of leukocytes to sites of inflammation. This also holds true in the context of lymphocyte recruitment and trafficking of these cells along the activated endothelium (Norling *et al.*, 2008).

Along with increased neutrophil recruitment in Gal-1 KO mice significant increases in the pro-inflammatory cytokine IL-6 as well as the pro-angiogenic growth factor VEGF were also detected. IL-6 is a pleiotropic cytokine involved in the acute phase response

and in the context of zymosan peritonitis inhibition of this cytokine reduces the inflammatory response (Cuzzocrea *et al.*, 1999). VEGF is also involved in the rapid recruitment of neutrophils *in vivo* (Massena *et al.*, 2015), hence aligning with elevated levels in Gal-1 null mice. The chemokines CCL2 (MCP-1), CCL3 (MIP-1α) and CXCL1 (KC), as well as the pro-inflammatory cytokine TNF-α and the growth factor GM-CSF also all revealed a trend towards increased levels in the absence of Gal-1, thus corroborating the heightened inflammatory response. The differences described above in the cytokine profile of Gal-1 KO mice compared to WT at 2h post zymosan suggest that a lack of Gal-1 may impact downstream signalling of TLR2 in macrophages.

The augmented neutrophil response at the peak (6h) did not have a notable impact on recruitment of any other leukocyte subsets to the peritoneal cavity during peak infiltration. Although significantly more neutrophils were found within the peritoneal cavity of Gal-1 KO mice at 6h, this difference was lost at the 24h time-point suggesting that clearance might be faster in these mice.

#### *4.1.2. Gal-1 Null Macrophages Exhibit Alternative Activation and Augmented Efferocytosis.*

To investigate the role of endogenous Gal-1 in neutrophil clearance, levels of apoptosis and efferocytosis were assessed from peritoneal exudates collected from multiple time points. As expected during the initiation of inflammation (2h) and resolving phase (24h and 48h), exudated neutrophils were predominantly viable, whereas at the peak (6h), the majority were early apoptotic, however there was no significant difference between genotypes. A trend towards increased early apoptotic

cells was detected at 24h in Gal-1 KO mice along with an increased percentage of efferocytic macrophages (F4/80+Ly6G+). However, these permeabilised macrophages had a lower Ly6G MFI suggesting they had ingested fewer neutrophils per macrophage than WT. Thus, whilst there were more macrophages clearing apoptotic neutrophils in Gal-1 KO mice the cumulative effect on clearance was likely similar between genotypes. It is feasible that the apoptotic/efferocytic process is faster or earlier in Gal-1 KO mice, or alternatively these results may simply be a reflection of the efficiency of the resolution process and the ability of the system to regain homeostasis.

Following ingestion of apoptotic neutrophils, macrophages swiftly depart the inflammatory site making efferocytosis a tricky process to capture *in vivo*. Studying the cells *ex vivo* can provide an insight, which cannot be obtained using this *in vivo* model. Interestingly, when inflammatory macrophages collected from Gal-1 KO mice were cultured *ex vivo* the cells exhibited an enhanced phagocytic capacity (Barrionuevo *et al.*, 2007), the same study also revealed significantly higher levels of MHC-II. In support of this latter finding, bone marrow derived macrophages from Gal-1 KO mice showed elevated MHC-II expression on M1 as well as M2 cells. BMDM from Gal-1 KO mice were consistently more ‘M2’ like and displayed significantly higher CD206 and lower CD86 than WT cells. Levels of the scavenger receptors CD36 and MerTK, which promote efficient phagocytosis also showed a trend for increased expression on Gal-1 null macrophages. These findings seem paradoxical when exogenous Gal-1 has been shown to promote macrophage conversion to a pro-resolving ‘M2’ phenotype (Rostoker *et al.*, 2013) and thus further illustrate the divergent roles of intracellular versus extracellular Gal-1.

It has already been established that macrophages are a major source of endogenous Gal-1 (Rabinovich *et al.*, 1996, 1998). Resident peritoneal macrophages exhibit scant levels of Gal-1, however *in vivo* elicited-inflammatory macrophages and *in vitro* activated macrophages have far greater levels of Gal-1 with expression in the nuclei, secretory granules as well as adjacent to the granules within the cytoplasm (Correa *et al.*, 2003). We demonstrated that Gal-1 was readily detected in peritoneal lavage fluid from mice undergoing zymosan peritonitis, with the highest levels at 48h. Our results corroborated published data; resident macrophages had very little Gal-1 expression whereas resolution phase macrophages (48h time point) had maximal expression (Law *et al.*, 2020). These results implicate another cellular source for Gal-1 in the peritoneum under a non-inflamed state.

Exogenous Gal-1 enhances macrophage infiltration into the resolving peritoneum (Gil *et al.*, 2006; Malik *et al.*, 2009). Thus, it was unexpected that increased numbers of macrophages were detected in the resolving (48h) cavity of Gal-1 null mice, specifically in the resolving ($CD11b^{low}$) population of macrophages with no difference detected in numbers of mature ($CD11b^{high}$) macrophages. Interestingly, endogenous Gal-1 has been shown to be selectively expressed by $CD11b^{high}$ macrophages and not by $CD11b^{low}$ macrophages (Rostoker *et al.*, 2013). In the same study it was reported that exogenous Gal-1 directly promoted macrophage conversion from a $CD11b^{high}$ to a $CD11b^{low}$ phenotype and subsequent efferocytic satiation. This suggests that Gal-1 in the inflammatory milieu is important for active resolution of inflammation. It could be the case that Gal-1 presence in the inflammatory milieu, either via the release of endogenous Gal-1 from $CD11b^{high}$ macrophages or from the

addition of exogenous protein, has a role in promoting a more rapid conversion of macrophages to a CD11b$^{low}$ pro-resolving phenotype which subsequently reach efferocytic satiation quicker and thus will have departed from the resolving cavity earlier. If this theory were to be the case the higher number of resolving macrophages found within the cavity of Gal-1 null mice during the resolution phase could be explained by a delayed conversion to CD11b$^{low}$ cells. However, a full characterization of Gal-1 KO macrophage phenotype has not been performed and as such the exact role of endogenous Gal-1 in macrophages still requires full examination.

Initially it was compelling to think that the increased number of macrophages detected in the Gal-1 KO mice at 48h was simply the direct response to the increased neutrophil infiltrate observed in these mice. However, further consideration of the data resulted in a revision of this hypothesis. Neutrophil numbers within the cavity were decreased at 24h, at which point they had also ceased to be increased in Gal-1 KO mice. There was also no increase in macrophages detected at 24h nor any suggestion of differences in efferocytosis at 48h. Taken together it seems highly unlikely that these CD11b$^{low}$ macrophages have been retained within the cavity because they are still required for the clearance of apoptotic neutrophils.

Protocols to detect apoptosis and efferocytosis in the zymosan-induced peritonitis model have been optimised, however these events remain very difficult to capture in *in vivo* models. Exposure of phosphatidylserine (PS) on the neutrophil surface occurs as part of the initial stage of apoptosis (Denecker *et al.*, 2000) and provides a rapid 'eat me' signal to macrophages. Following their recognition of this signal macrophages are quick to respond and rapidly begin to engulf these PS exposing early apoptotic

cells. The percentage of cells that remain in the cavity following progression to late apoptosis are minimal. As such it was decided to assess neutrophil apoptosis *ex vivo* comparing genotypes. Significantly more apoptotic neutrophils were detected from peritoneal exudates collected at 6h from Gal-1 KO mice compared to WT. This suggests that apoptosis might progress faster in the absence of Gal-1, or it may be a reflection of increased neutrophil numbers within the peritoneal cavity at this time point. Regarding the latter there are a number of published studies supporting a positive correlation between cell density and spontaneous apoptosis and that this is the case for both adherent cells and cells cultured in suspension (Saeki *et al.*, 1997; Qiao and Farrell, 1999). In this study cells from 1ml of lavage fluid collected from each mouse were resuspended in low serum media, plated and cultured overnight to undergo apoptosis. As cell number was not normalised, there would have been more neutrophils collected and plated from Gal-1 KO mice than WT, a difference which may well have impacted the rate of apoptosis and therefore could explain the significant increase in the *ex vivo* apoptosis of neutrophils from Gal-1 KO mice. In recognition of this discrepancy it would be pertinent to determine if apoptosis of viable neutrophils taken from Gal-1 KO mice would still progress quicker if peritoneal neutrophils were at the same cell density as WT cells. An additional important consideration regarding this *ex vivo* assay is the different levels of inflammatory mediators present *in vivo* within the peritoneal cavity of Gal-1 KO mice compared to WT. When measured during the early phase of the inflammatory response in this model Gal-1 KO mice were shown to have comparatively elevated levels of several inflammatory mediators, including GM-CSF, known to influence neutrophil survival. Thus, it is worth considering that the loss of pro-survival signal, resulting from *in vivo* to *ex vivo* transition, was likely greater for Gal-1 KO cells and potentially impacted on their rate of spontaneous apoptosis.

### *4.1.3. Exogenous Gal-1 Reduces Neutrophil Number at the Inflammatory Site when Administered at the Peak of Inflammation.*

Both IL-1β and zymosan-induced peritonitis stimulate an acute immune response within a few hours post injection into the peritoneal cavity. The similarities between the models and previous findings seen in IL-1β induced peritonitis led to the hypothesis that neutrophil numbers within the peritoneal cavity would decrease in response to treatment with hrGal-1 in this *in vivo* model of zymosan induced peritonitis. However, despite that both studies assessed cellular infiltrate into the peritoneal cavity four hours post administration of hrGal-1 the same outcome was not reflected in this experimental setting. Indeed, it could be the case that the current study showed no significant modification of the leukocyte numbers in response to hrGal-1, when given early during the recruitment phase, due to the mechanistic differences between the models. In peritonitis induced by IL-1β there is direct activation of the endothelial cells whereas in the zymosan model the inflammogen is acting via toll like receptors (TLR) on macrophages. The mechanistic differences stimulating the inflammatory response in each of these *in vivo* models may be accountable for the differences seen in neutrophil trafficking and recruitment.

The disparity between models may also be due to timing of administration of the recombinant protein or the dosing strategy utilised for the administration of hrGal-1 in this study. Previous *in vitro* studies demonstrating an inhibitory effect on neutrophil recruitment have involved pre-incubating neutrophils with hrGal-1 prior to use in an *in vitro* flow chamber model (Cooper *et al.*, 2008). In the IL-1β–induced peritonitis model hrGal-1 was co-administered with the pro-inflammatory stimulant (La *et al.*, 2003). Whereas in this current study, therapeutic administration of hrGal-1 was given two

hours post-stimuli, when the inflammatory response had already been initiated. In this case rapid recruitment of neutrophils towards the peritoneal cavity would have already taken place at the time of hrGal-1 treatment and thus might account for the lack of effect in this system. Future *in vivo* studies should consider the co-administration or prophylactic treatment with hrGal-1 in the Zymosan peritonitis model to verify that there is truly no difference in leukocyte and more specifically neutrophil recruitment in response to exogenous hrGal-1.

Aside from the neutrophil population consideration should also be given to macrophages which have a key role in facilitating the inflammatory response. Few studies have investigated how Gal-1 may influence the role of macrophages in the initiation of the inflammatory response, with studies having focused primarily on macrophages during the resolution phase. In this scenario it has been indicated that Gal-1 promotes an anti-inflammatory pro-resolving phenotype in macrophages (Rostoker *et al.*, 2013), a finding which will be elaborated on in a more relevant context later in this discussion.

Although the literature is comparatively limited it is not unresearched and *in vitro* findings on Gal-1 treated macrophages showed inhibited arachidonic acid and $PGE_2$ secretion in response to LPS stimulation (Rabinovich *et al.*, 2000). Furthermore, a study utilising activated peritoneal macrophages revealed that Gal-1 inhibits nitric oxide (NO) production and expression of inducible NO synthase (iNOS) in these cells instead favouring arginase activity (Correa *et al.*, 2003). Taken together these findings indicate that Gal-1 promotes macrophage preference towards taking the alternative metabolic pathway consequently downregulating the classical metabolic pathway. In

addition, Gal-1 reduces macrophage production of pro-inflammatory cytokines including IL-1β, IL-6 and the chemokine MCP-1 (Correa *et al.*, 2003; Kogawa *et al.*, 2011; Abebayehu *et al.*, 2017). Consequently, it could be predicted that the pro-inflammatory response following macrophages being stimulated with an inflammogen such as zymosan, may be dampened in the presence of Gal-1 however there was no evidence from the recruitment of leukocytes that this was the case in this study.

Importantly, in this study neutrophil numbers in the peritoneal cavity were determined following exogenous Gal-1 administration during the peak of inflammation (at a time point that should not affect neutrophil recruitment) in order to assess neutrophil clearance. Administration of hrGal-1 at this time-point resulted in significantly fewer leukocytes being recovered 16h later (24h time point) compared to those recovered from vehicle treated mice. *In vitro* studies from Stowell *et al* describe the ability of Gal-1 to induce exposure of the “eat me” signal PS on the surface of neutrophils (Stowell *et al.*, 2007, 2009). Interestingly, PS exposure normally occurs as part of the early stages of apoptosis, however Gal-1 was not found to induce apoptosis in their studies, rather it was proposed that Gal-1-induced PS exposure is a mechanism to drive clearance of neutrophils in the absence of apoptosis. Here, increased numbers of PS exposing ‘early apoptotic’ neutrophils were detected within the peritoneal cavities of mice treated with hrGal-1. Whilst it was not possible to determine whether these cells progressed through the apoptotic process presumably due to the rapid clearance of PS expressing cells *in vivo*, we did uncover a pro-apoptotic role for Gal-1 when assessing the stages of apoptosis *ex vivo*.

*4.1.4. The Resolution Interval is Reduced by Exogenous Gal-1.*

Pro-resolving mediators are typically produced at the peak of the inflammatory response as they function to halt leukocyte infiltration and to promote active clearance of the cellular infiltrate thus allowing a return to homeostasis. Typically, proteins/peptides/lipids are administered at the peak of inflammation to assess their impact on the resolution interval, as pioneered by the Serhan laboratory (Bannenberg *et al.*, 2005). When Gal-1 was administered at the 8h time point, the resolution interval was shortened from 39 to 14h, suggesting that Gal-1 has pro-resolving properties. As neutrophil numbers are highest when Gal-1 was administered this implies that it has an action on their clearance rather than neutrophil recruitment in this experimental setup.

Analysis of the individual leukocyte subtypes indicated that there was a trend towards an all-round decrease in the numbers of all leukocyte subsets in hrGal-1 treated mice with significantly fewer macrophages found to be within the cavity of hrGal-1 treated mice. This data is in line with the increased macrophages present within the cavity in Gal-1 KO mice. Interestingly, the absence of the endogenous protein specifically altered resolving ($CD11b^{low}$) macrophage numbers whereas the addition of the exogenous protein influenced primarily mature ($CD11b^{high}$) macrophages with a lesser effect on resolving macrophages. The aforementioned study by Rostoker *et al*., demonstrating that exogenous Gal-1 promoted the conversion of macrophage phenotype from $CD11b^{high}$ to $CD11b^{low}$ (Rostoker *et al.*, 2013), may provide an explanation for the data presented here. Specifically, if macrophages were converted from $CD11b^{high}$ in response to hrGal-1 it would account for fewer of these cells remaining in the system. The fact that there are not higher numbers of $CD11b^{low}$

macrophages detected as a result of this is not unexpected given that these cells, with their pro-resolving phenotype, are swift in their response to PS exposing cells and following efferocytosis depart from the site. Of relevance to this current research it was also reported in the Rostoker *et al*., study that Gal-1 treatment *in vivo* promoted efferocytic satiation being reached more quickly in these $CD11b^{low}$ macrophages and thus a loss of their phagocytic capacity (Rostoker *et al.*, 2013). This concept provides further support for the more rapid neutrophil engulfment by $CD11b^{low}$ macrophages and their subsequent departure as suggested by the data from the present *in vivo* study. Together these findings indicate that the endogenous and exogenous protein have disparate roles during the resolution of inflammation and that the capability of Gal-1 to facilitate actions on distinct macrophage populations may underline the proteins ability to resolve inflammation.

When trying to develop a better understanding of the role of hrGal-1 in resolution and the mechanistic actions promoting cell clearance it is essential to also consider the neutrophil population. In this study neutrophils post-zymosan injection were further assessed for apoptosis. Most neutrophils were viable presumably as a result of the rapid clearance of any apoptotic cells. Importantly, significantly fewer viable cells were detected within the peritoneal cavities of mice treated with hrGal-1 and a significant increase in the percentage of early apoptotic neutrophils ($AnxV^+/PI^-$) was observed. The increase in PS positive cells in response to hrGal-1 detected in this model is corroborative with existing data in the literature. As previously mentioned, a study by Stowell *et al.,* revealed that hrGal-1 induced PS exposure on the cell surface, furthermore it was also proposed that this occurs in the absence of apoptosis, a process termed as “preaperesis” as a mechanism to assist the progress of

efferocytosis (Dias-Baruffi *et al.*, 2003; Stowell *et al.*, 2007, 2009). However, the nature of this *in vivo* model with the rapid clearance of PS exposing cells made it impossible to determine if this were the case or if the PS positive neutrophils were in fact in early apoptosis and would be detectable as having progressed to late apoptosis in the absence of macrophages.

Therefore, to further address the effect of exogenous Gal-1 on the apoptotic process, hrGal-1 was administered during the initial recruitment phase (2h) and peritoneal infiltrate collected during neutrophil peak (6h). Cells were then incubated *in vitro* in low serum media overnight to undergo apoptosis. In this *ex vivo* setting it was found that neutrophil progression to late apoptosis (AnxV+PI+), as detected by positive PI staining indicating a loss of membrane integrity, was significantly increased following exposure to hrGal-1. In contrast to the results seen by Stowell *et al.* these findings indicate that hrGal-1 induced PS exposure is coupled with the progression of apoptosis. This discrepancy between the studies could be explained by the timing of cell assessment, amount/concentration of hrGal-1, cell type and/or activation state and will be discussed more elaborately in the context of the *in vitro* assays on human neutrophil survival performed as part of the scope of this thesis.

#### *4.1.5. Exogenous Gal-1 Showed no Effect on Delayed Self-resolving Inflammation.*

Curious to determine if Gal-1 restores resolution in a delayed self-resolving model of peritonitis, high dose (10mg) zymosan was utilised. Mice were sacrificed at 24h to obtain ‘baseline’ values and the remaining mice received either hrGal-1 (10µg) or vehicle only by daily i.p. injection. To profile the response across time mice were

sacrificed at 72h and 120h. There was no significant effect of hrGal-1 in this experiment.

These results suggest that the protein may have to come into direct contact with the recruiting cell or endothelium to have an effect on inhibiting recruitment as seen in other models (La *et al.*, 2003) or that is may have to be co-administered or given prophylactically to observe its effect on recruitment.

Although disappointing that these results did not yield the same enhanced resolution profile in response to hrGal-1 as seen in the acute self-resolving model, there may still have been enhanced neutrophil apoptosis and the macrophage phenotype switching of the cells within the cavity. The administration of a higher dose of inflammogen results in prolonged recruitment of neutrophils to the peritoneal cavity in comparison to the acute model. This persistent emigration of new cells would ensure a constant supply of viable neutrophils and therefore it is plausible that any effect of hrGal-1 on inducing apoptosis and enhancing clearance of these short-lived cells may be masked by the excessive and persistent influx of new cells.

In line with the above theory the percentage of viable neutrophils (from treated and non-treated mice) was higher in this system than in the acute model. In support of the potential enhanced clearance in response to hrGal-1 there was a reduced percentage of apoptotic neutrophils detected in the peritoneal cavity at 120h in hrGal-1 treated mice, as well as reduction in number of resolving macrophages, suggesting that apoptotic neutrophils have been cleared and that the efferocytic macrophages have departed the cavity. Furthermore, the data from 72h evidenced a subtle increase in

resolving macrophage numbers and apoptotic neutrophils in Gal-1 treated mice. More convincingly when assessed for efferocytosis the percentage of macrophages positive for neutrophils was higher in hrGal-1 treated mice at this time point.

To summarise, it could be speculated that hrGal-1 still induces PS exposure on neutrophils resulting in enhanced clearance taking place between the 72h and 120h time point, yet this effect is masked by the stream of new viable neutrophils entering the cavity in replacement of their short lived predecessors and in an attempt to maintain control of this more severe inflammatory system.

It would also be interesting to determine if there was any effect of endogenous Gal-1 in this model by utilising the Gal-1 KO mice. However due to the heightened inflammatory response that was seen in the WT mice, it may not be a suitable experiment to perform in KO mice on the basis of their increased recruitment in the acute model.

#### *4.1.6. Summary.*

The divergence in the effects of exogenous, extracellular Gal-1 and the intracellular protein make it difficult to compare the effects seen in global knockout mice with studies that have utilised the recombinant protein. Furthermore, previous data from our laboratory suggest that compensatory mechanisms may exist in Gal-1 KO mice (Iqbal *et al.*, 2011). None the less these findings confirm an anti-trafficking role for endogenous Gal-1 (figure 63A) and indicate a pro-apoptotic function for the exogenous soluble protein (figure 63B).

The clearance of apoptotic cells is a fundamental process following cell damage, ageing and trafficking of leukocytes to an inflammatory site and hence is critical for the resolution of inflammation and preservation of self-tolerance. The reduction in the resolution interval in response to Gal-1 in this model provides further indication that the protein augments the transition from inflammation to resolution. Gal-1 administration at the 8h time point reduced the resolution interval from 39h to 14h, suggesting that Gal-1 has pro-resolving properties that may be of therapeutic utility.

Overall results from this *in vivo* model enhance our understanding of the role of Gal-1 in resolution and shed light on the promising nature of this protein for the development of a new class of therapeutics that activate resolution.

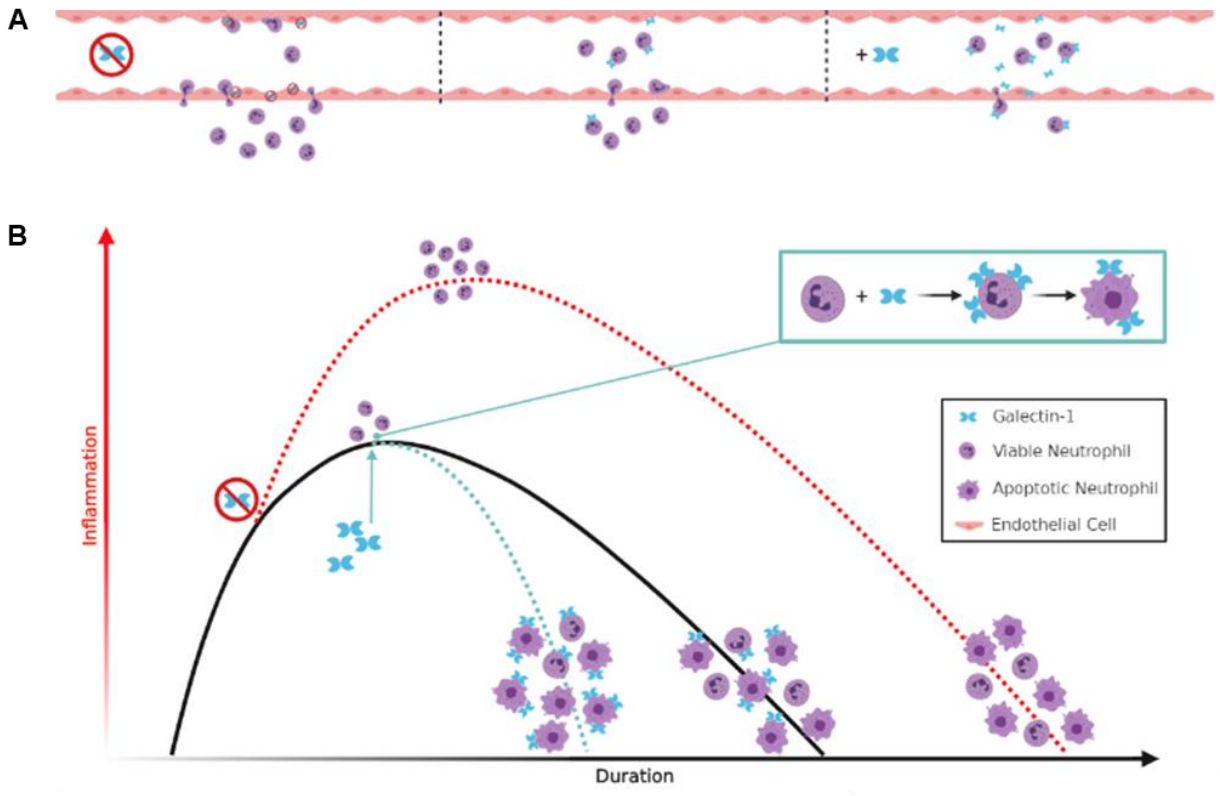



**Figure 63. A pro-resolving role for Gal-1 in acute inflammation.**
Mice received zymosan (1mg i.p.) to elicit an acute self-resolving inflammatory response and peritoneal lavage was performed to examine cellular infiltrate to the site of inflammation. During the early phase, the absence of endogenous Gal-1 resulted in increased neutrophil recruitment from the vasculature to the site of inflammation whereas administration of exogenous Gal-1 reduced the number of trafficked neutrophils (**A**). Administration of exogenous Gal-1 during the peak of inflammation enhanced neutrophil apoptosis and thus reduced neutrophil numbers and shortened the resolution interval (**B**). Taken together these results indicate important pro-resolving actions for Gal-1 in the timely resolution of acute inflammation. [Created using BioRender illustrating tool.]

### *4.2. Gal-1 Influences the Disease Profile in a Model of Rheumatoid Arthritis.*

#### *4.2.1. Disease Progression and Resolution are Augmented in Gal-1 Null Mice.*

In this current study Gal-1 KO mice developed arthritis at a slower rate, taking longer to reach the peak of their disease, as evidenced by clinical score, weight loss and oedema. WT mice lost significantly more weight and had significantly increased oedema compared to their knockout counterparts. The lessened disease in the absence of Gal-1 was corroborated by the significantly decreased cellular infiltrate in the knee when analysed microscopically by histology and a more subtle decrease in neutrophil numbers in the paw when analysed by flow cytometry.

Interestingly there was a significant increase observed in the fast response paw oedema in Gal-1 KO mice. This initial inflammation that shortly follows the administration of K/BxN serum is driven by mast cells (Corr and Crain, 2002), which degranulate as early as 1h after intraperitoneal transfer of K/BxN serum, thus preceding the onset of clinical signs of inflammation (Lee *et al.*, 2002). This early response has been likened to an Arthus reaction (Corr and Crain, 2002), indicative of a local type III hypersensitivity reaction involving the deposition of immune complexes initiating an inflammatory response that will then progress to drive pathogenesis of tissue injury (Sylvestre and Ravetch, 1994). The detection of immune complexes in human rheumatoid arthritis synovial fluid provides further evidence to the relevance of this response (Mathsson *et al.*, 2006; Monach *et al.*, 2009; Weissmann, 2009). This reaction to delivery of K/BxN serum is regularly encountered in experimental settings following the injection of autoantibodies so it is not unexpected that it functions in this model. Alongside immune complexes, mast cells have been demonstrated to play a

key role in the Arthus reaction (Sylvestre and Ravetch, 1996) and are known to be present in the synovium of mice. Furthermore, their importance in this model is evidenced by diminished arthritis in mice lacking tissue mast cells (Hayashi *et al.*, 1985; Huang *et al.*, 1990; Corr and Crain, 2002).

Current studies regarding Gal-1 interplay with mast cells have suggested that Gal-1 downregulates recruitment and degranulation of mast cells (Rabinovich *et al.*, 2000; Xie *et al.*, 2017). Therefore, it is plausible that in the absence of Gal-1 these processes are elevated and as such are accountable for the significantly enhanced mast cell driven response in the Gal-1 KO mice seen here. Furthermore a study looking at uterine mast cells in the context of embryo implantation revealed that mast cells are capable of secreting endogenous Gal-1 (Woidacki *et al.*, 2013), However, whether this ability is shared by mast cells at inflammatory sites and what effect it may have in this environment is yet to be determined.

Mast cells have been proposed to function as an important cellular link between autoantibodies, soluble mediators and other effectors in inflammatory arthritis (Lee *et al.*, 2002). Aside from their aforementioned link to immune complexes and their degranulation, the latter potentially modulated by Gal-1, stimulated mast cells present at the inflammatory site also release leukotriene $B_4$ ($LTB_4$), a potent neutrophil chemoattractant (Malaviya and Abraham, 2000; Miyahara *et al.*, 2009). Although plasma levels of this lipid mediator were not significantly reduced in Gal-1 KO mice this does not necessarily imply that levels at the inflammatory site would also be indifferent in the absence of Gal-1. It could be the case that the significantly increased oedema in Gal-1 KO resulting from the early activation and degranulation of these

cells in response to transfer of K/BxN serum, influences mast cell survival, phenotype and/or inflammatory environment in such a way to impair the actions of $LTB_4$. Specifically, a reduction in the release of $LTB_4$ or a dampening of its chemoattractant effect within the inflammatory milieu would subsequently impact on neutrophil recruitment to the inflammatory site and thus provide explanation for the lessened disease seen in the Gal-1 KO mice. Although assessment of neutrophil percentage at day 4 indicated no significant alteration in the absence of Gal-1, this analysis was performed without total cell enumeration and thus the data does not reveal whether there was an altered number of total CD45+ cells within the joint. Indeed, a possible explanation for the decreased infiltrate detected by histology or the lessened inflammation and disease detected in the absence of Gal-1 could be ascribed to the role of mast cell leukotrienes in neutrophil recruitment.

Significant differences were also detected for the T cell populations within the draining lymph nodes collected from Gal-1 KO mice compared to WT. Here it was shown that in the absence of Gal-1 a significantly lower percentage of lymphocytes were CD8+ whereas CD4+ cells were slightly increased with a notable shift in the phenotype of these cells. The decrease in CD8+ cells in the KO compared to lymph nodes from WT mice is in line with evidence that patients with active rheumatoid arthritis have increased numbers of CD8+ T cells within the draining lymph node and that this is likely due to their recirculation to the inflamed synovium (Kondratowicz *et al.*, 1990). Indeed, as a result of their activated effector memory phenotype, preferential homing to the inflamed tissue and subsequent production of proinflammatory cytokines, CD8+ lymphocytes have been shown to have an essential role in maintaining chronic synovitis in the K/BxN mouse model (Raposo *et al.*, 2010).

CD4+ T cells are known to have broad spectrum local and systemic roles in the induction, regulation and maintenance of inflammatory joint disease (as reviewed by (Alzabin and Williams, 2011)). In K/BxN mice the CD4+ T cells specific for recognising the self-protein glucose-6-phosphate isomerase (G6PI) are essential for initiating joint pathology (Matsumoto and Staub, 1999). Furthermore when tissue resident these cells have an activated state inferring their role in disease (Hovdenes *et al.*, 1989). In this case the reduced inflammation in Gal-1 KO mice, could be an indication of a reduced population of inflammatory lymphocytes residing in the joints, thus increased detection of CD4+ lymphocytes in the draining lymph node may be due to their departure from the inflammatory site.

Importantly, lymphatic drainage to the lymph node has been proposed to have a protective effect on synovitis, bone erosion and cartilage loss and evidence implicates a fundamental role for VEGF in controlling lymphatic drainage (Guo *et al.*, 2009; Zhou *et al.*, 2011). In line with this it has been shown that VEGF and the receptors VEGFR-2 and VEGFR-3 are abundantly expressed in the blood vessels of the synovial sublining in rheumatoid arthritis (Paavonen *et al.*, 2002). Interestingly, Gal-1 has been shown to bind to VEGFR-2 receptor (Hsieh *et al.*, 2008). However, studies on this interaction have focused primarily on the tumour microenvironment, where Gal-1 acts as an alternative ligand when anti-VEGF treatments are applied (Croci and Rabinovich, 2014). Whether Gal-1 interacts with VEGFR on endothelial cells and modulates its effects in the context of inflammation are currently unreported. Increased levels of VEGF were detected in Gal-1 KO mice in the acute peritonitis model, suggesting that modulation of the growth factor and/or receptor(s) occurs in the

absence of Gal-1. Further investigation is required to determine the role of cellular glycosylation signatures on the angiogenesis and the vasculature in response to Gal-1, however it appears to be an intriguing area of research (Croci *et al.*, 2014).

An alternative and more probable possibility for the increased number of effector T cells seen in the absence of Gal-1 correlates with well-established reports in the literature of the ability of Gal-1 to induce the apoptosis of effector T cells. Specifically, Gal-1 expressed by endothelial cells and stromal cells has been shown to induce the apoptosis of T cells bound to the endothelium and those present in the thymus and lymph nodes respectively (Perillo *et al.*, 1995). In addition to the increased population of these effector T cells in the absence of Gal-1, consideration should also be paid to their role within this model of arthritis. Of the CD4+ population of lymphocytes a significantly increased percentage of effector ($CD62L^{-}CD44^{-}$) T cells were seen in the lymph nodes from Gal-1 KO mice whereas the naive ($CD62L^{+}CD44^{-}$) T cell population was significantly decreased. Importantly, CD4+ effector cells can differentiate into many phenotypes, broadly speaking these fall in to four main categories: those with pro-inflammatory effector characteristics, those with regulatory/anti-inflammatory actions, those that promote B cell follicle development and those that provide long term memory (Eagar and Miller, 2019). The lessened disease evidenced in the Gal-1 KO mice compared to WT would suggest expansion of the population of effector T cells that have a regulatory/anti-inflammatory phenotype in the absence of Gal-1.

On the basis that naïve T cells are precursors for differentiation into effector and memory T cell subsets the significant increase in effector T cells and the slight increase in memory T cells likely serves as the explanation for the corresponding decreased

population of naïve T cells in the draining lymph nodes from Gal-1 KO mice. Naive T lymphocytes are activated through their T cell receptors (TCRs) by peptide–MHC complexes, and only upon activation, undergo rapid proliferation differentiating into effector cells (Catron *et al.*, 2006). MHC class II (MHC-II) molecules expressed on dendritic cells, monocytes and macrophages present antigen to CD4+ T cells (Holling *et al.*, 2004), an essential interaction for the initiation of an antigen-specific immune response. As part of the scope of this thesis MHC-II expression was assessed on (bone marrow derived) macrophages from WT and Gal-1 KO mice, here it was shown that expression was higher on macrophages absent of Gal-1, a result which corroborates with existing literature (Barrionuevo *et al.*, 2007). It could be postulated that the enhanced MHC-II expression seen in Gal-1 KO mice could directly encourage the differentiation of naïve T cells to effector T cells as a result of the increased antigen presentation during the inflammatory response in these mice.

The delayed progression of disease observed in Gal-1 KO mice in this model of K/BxN serum transfer induced arthritis was contrary to expectation and indicates a divergence for the role of the endogenous Gal-1 in neutrophil-driven pathologies compared to T cell-driven models, such as collagen-induced arthritis (CIA). Gal-1 KO mice exhibit accelerated onset and increased severity of disease compared to WT mice in a model of CIA, suggesting that endogenous Gal-1 acts to dampen the T cell activation driving disease (Iqbal *et al.*, 2013). Conversely, in this current study absence of Gal-1 does not increase either the rate of disease onset or the maximum clinical severity in a neutrophil-driven model. This suggests that the effect of Gal-1 and its role in inflammatory pathologies is likely dependent on the cellular mechanisms initiating and driving disease progression. In line with this Gal-1 KO mice also displayed a

reduced response to inflammatory stimuli in a model of carrageenan induced paw oedema (Iqbal *et al.*, 2011). The disease profile of this model displays a biphasic response, in support of what was seen in the K/BxN arthritis model presented here the immediate response to administration of stimuli evidenced increased oedema in the absence of Gal-1. Furthermore, during the second phase of the response the intensity of the oedema was significantly lower in the Gal-1 KO mice compared to WT, as was found in this study for the progression of arthritis. Analysis of the leukocyte infiltrate in the paw oedema model revealed a reduced infiltration and increased apoptosis of neutrophils in the Gal-1 KO mice. These findings appear to conflict with the anti-inflammatory properties reported for Gal-1 (Norling *et al.*, 2009) which may suggest that other pathways and/or mediators are involved in the inflammatory response in these models. It was found in the paw oedema model that Gal-9 mRNA expression significantly increased in Gal-1 KO mice at the onset of resolution indicating potential compensatory mechanisms between galectins in these global knockouts (Iqbal *et al.*, 2011). This increase in mRNA, although absent of any effect at the level of protein expression, indicates a potential interplay between members of the galectin family with endogenous roles in inflammation and provides further evidence that levels of endogenous galectins are modulated at different phases of the inflammatory response.

As was observed in the model of zymosan-induced peritonitis, remission indices in the K/BxN model of arthritis were increased in Gal-1 KO mice compared to WT, suggesting again that Gal-1 has a pro-resolving function. Interestingly, the potential pro-resolving role of Gal-1 was also apparent in the model of paw oedema, with increased expression at both the gene and protein level detected in WT mice at the later time

points, with peak expression correlating with the onset of resolution (Iqbal *et al.*, 2011). In line with this theory and suggestive of the translational relevance of these findings, Gal-1 levels are significantly reduced in the synovial fluid from rheumatoid arthritis patients compared to healthy controls (Mendez-Huergo *et al.*, 2019). A corresponding increase in the titres of anti-Gal-1 autoantibodies has been proposed to be responsible for limiting the amount Gal-1 and thus blocking its biological effect (Mendez-Huergo *et al.*, 2019). As such the pro-resolving function of Gal-1 could be limited in arthritic joints, which may in turn contribute to the uncontrolled inflammatory responses driving chronic disease (Xibillé-Friedmann *et al.*, 2013). Still, additional findings suggest a divergence in the role of the protein dependant on its localisation.

Interestingly, levels of Gal-1 detected in the serum of rheumatoid arthritis patients has been shown to be significantly higher compared to healthy controls, correlating with disease activity (Vilar *et al.*, 2019). Here it could be suggested that the elevated Gal-1 levels in the peripheral blood are detected due to increased release of the endogenous protein from the chronically activated endothelium as has previously been revealed (Baum, Seilhamer, *et al.*, 1995). One theory would be that release of endogenous Gal-1 is upregulated in a physiological process by endothelial cells in their attempt to inhibit aberrant leukocyte trafficking and regain control of recruitment to the inflamed site. However, it is also a possibility that the high levels of Gal-1 found in the sera of the rheumatoid arthritis patients is directly due to the protein having a role in the anti-inflammatory effects of the DMARDS and corticosteroids that have been used to treat the inflammation in these patients (Mendez-Huergo *et al.*, 2019). In any case, aside from the modulated expression the precise mechanism(s) behind the anti-inflammatory and/or pro-resolving effect of Gal-1 is yet to be elucidated.

### *4.2.2. Local Administration of Exogenous Gal-1 did not Modulate the Course of Arthritis.*

In this current study local therapeutic treatment with exogenous Gal-1 from clinical onset of disease had no significant effect when assessed at peak (day 7) and resolving (day 11) inflammation. These findings were contrary to my prediction that an anti-inflammatory and/or pro-resolving role would be evidenced in this model in response to Gal-1.

Previous studies of exogenous Gal-1 were in the collagen-induced arthritis (CIA) model. In this model immunisation of mice with type II collagen (CII) resulted in development of arthritis that has both clinical and histological similarities to human rheumatoid arthritis (Brand *et al.*, 2004). The development of autoimmune polyarthritis in CIA is linked to MHCII and the immune response to CII, including stimulation of CII specific T cells and production of CII specific antibodies (Brand *et al.*, 2004). Similar to the K/BxN model, clinical onset of disease coincides with severe synovitis, mass accumulation of inflammatory cells (neutrophils and lymphocytes) and progresses to destruction of the joint. Despite the pathological similarity of the two models, both to one another and to human rheumatoid arthritis, there was significant disparity in the effect of exogenous Gal-1. In contrast to the findings obtained in this current study administration of rGal-1 was able to reduce disease severity in a model of CIA (Rabinovich, Daly, *et al.*, 1999). Two therapeutic protocols were used at onset of arthritis with both a gene therapy protocol (with a single injection of synovial fibroblasts expressing mGal-1) and a protein therapy protocol (injecting hrGal-1 (100μg) daily) successfully suppressing arthritis (Rabinovich, Daly, *et al.*, 1999). The diminished inflammation was suggested to be the result of a shift from a Th1 pathogenic response

towards a Th2 profile, impaired T cell function during antigen presentation and an increased susceptibility of T cells to apoptosis (Rabinovich, Daly, *et al.*, 1999).

The lack of effect of the exogenous protein in the current model strongly suggests divergence in the actions of Gal-1 on T cells compared to neutrophils, indicative of different roles for the protein in inflammation dependent on what is driving the response. Nonetheless, it has been shown that exogenous Gal-1 has the ability to induce apoptosis of neutrophils from synovial fluid of patients with rheumatoid arthritis (Cedeno-Laurent *et al.*, 2010), thus the lack of effect of the exogenous protein in this model was disappointing. It could be the case that the amount of protein administered in this study was insufficient to have a significant pharmacological effect in the K/BxN serum induced arthritis model. Here 3µg of hrGal-1, a dose that was shown to be effective in the carrageenan induced paw oedema model (Iqbal *et al.*, 2011), was administered. This dose was given therapeutically from established disease (day 3) by intraplantar injection into the right foot pad every alternate day. Indeed, the dosing strategy used in this current study differs in several aspects to the protein therapy protocol that was used in the CIA model where 100µg of hrGal-1 was given by daily intraperitoneal injection. The difference in route and amount of protein administered in the CIA model would allow for the protein to have a systemic effect, whereas the low dose at the site of inflammation used in the K/BxN model here provides for a local effect of the exogenous protein only, thus not accounting for the effect of Gal-1 on leukocytes trafficking along the endothelium (Baum, Seilhamer, *et al.*, 1995; Cooper *et al.*, 2008; Norling *et al.*, 2008). Furthermore, the study by Cedeno-Laurent et al., also used a notably higher amount of hrGal-1, specifically 0.25µM - 2.5µM (Cedeno-Laurent *et al.*, 2010), whereas the 3µg administered in the K/BxN model here is

equivalent to 214pM and is thus below the lowest concentration shown to induce the apoptosis of neutrophils from synovial fluid of rheumatoid arthritis patients. Thus despite its effective anti-inflammatory effects in the model of paw oedema (Iqbal *et al.*, 2011) perhaps in the K/BxN arthritis model 3µg was below the required amount for a significant pharmacological effect to be had in this chronically inflamed system.

The persistent infiltration and prolonged survival of neutrophils in the synovial fluid contributes to the pathogenesis of human rheumatoid arthritis and thus this aspect of the disease is an important target and further study is required to determine the role of the protein on neutrophils in the joint. Although findings from this study were not indicative of an effect of the exogenous protein in this model, additional assessment is required under adjusted parameters, including dosage and duration, to determine with more certainty whether an effect of the recombinant protein exists here. Previous studies in the CIA arthritis model have utilised more novel delivery mechanisms, including rGal-1 conjugated to gold nanoparticles (Huang et al., 2012) and intra-articular administration of lentiviral vectors (Wang et al., 2010), which in addition to direct protein administration have also evidenced reduced disease severity in response to exogenous Gal-1. It would be interesting to determine if these approaches have the same therapeutic effect in the K/BxN serum induced arthritis model.

Another important difference which may explain the lack of effect of exogenous Gal-1 in the K/BxN model is the experimental setting. In comparison to the CIA study the mice used for K/BxN serum induced arthritis are of a different strain, with DBA/1 mice used in the former and C57Bl6 in the latter. Interestingly, in both cases the genotype of mouse is selected for their response to disease induction, for example lymphocytes

from C57Bl6 mice fail to respond to the collagen peptide responsible for initiating disease in the CIA model, indicating differences in the T cell epitope specificities between the two strains (Inglis *et al.*, 2007). Likewise, DBA/1 mice showed a lesser response in the K/BxN model (Ji *et al.*, 2001). It is well established that during inflammation Gal-1 interacts with T cells, thus it is possible that the differences in the lymphocyte phenotype may account for the effectiveness of Gal-1 in the CIA model in DBA/1 mice and the lack of effect in the C57Bl6 mice in the K/BxN serum induced arthritis. Furthermore, the success of the exogenous protein in inducing apoptosis of rheumatoid arthritis synovial fluid neutrophils was shown in an *ex vivo* setting where leukocytes were collected and incubated with hrGal-1 for 12h/24h prior to analysis (Cedeno-Laurent *et al.*, 2010), consequently the levels of inflammatory cytokines and persistent infiltration of new leukocytes were not present in this system. It could be postulated that the inflammatory environment surrounding neutrophils *in vivo,* specifically within the inflamed synovium of the K/BxN model, provides the cells with pro-survival signals too strong for exogenous Gal-1 to overcome. Indeed, hrGal-1 successfully increased the rate of spontaneous apoptosis in the *ex vivo* study by Cedeno-Laurent et al., but whether this finding is translatable to an *in vivo* setting where pro-survival cytokines present in the inflammatory milieu afford neutrophils a prolonged lifespan requires further investigation. As such, the effect of hrGal-1 on the spontaneous apoptosis of neutrophils, in the presence of pro-survival cytokines has been studied as part of the scope of this thesis and will later be discussed in detail (see section 4.4).

*4.2.3. Summary.*

The divergence between the function of the intracellular, extracellular and exogenous forms of Gal-1 mean that the results obtained from global knockout mice may not be directly comparable with those that have used the recombinant protein. In most systems Gal-1 typically acts in an anti-inflammatory and/or pro-resolving manner and has been shown to curb the actions of both innate and adaptive immune responses. Gal-1 has been shown to dampen production of pro-inflammatory cytokines, inhibit neutrophil trafficking, modulate eosinophil migration and survival and reduce mast cell degranulation and is thus considered to act as a resolution associated molecular pattern (RAMP) in the innate response (Shields *et al.*, 2011; Sundblad *et al.*, 2017). In the context of the adaptive immune response Gal-1 has been shown to act at a regulatory checkpoint and modulate receptor clustering and signalling on T and B cells, as well as affect the recruitment and survival of activated T cells. Certainly, these actions would suggest that harnessing the properties of Gal-1 might provide a mechanism to regulate the inflammatory response, offering a promising therapeutic strategy for chronic autoimmune inflammatory diseases such as rheumatoid arthritis.

The delayed remission of disease observed in the absence of endogenous Gal-1 in this model strongly suggests a role for the protein in the resolution process. Likewise results obtained from the zymosan peritonitis model of acute self-resolving inflammation, demonstrated a marked reduction in the resolution interval in response to therapeutic treatment with hrGal-1 from the peak of inflammation. Taken together these findings support a pro-resolving role for Gal-1 by enhancing neutrophil clearance from the site of inflammation and indicate that the protein influences the inflammatory

profile in a model of rheumatoid arthritis, consequently reinforcing the therapeutic potential of this lectin in clinical settings.

### *4.3. Macrophage Efferocytosis was not Altered by the Absence of Gal-1.*

Assessment of efferocytosis *in vivo* in the absence of inflammatory challenge revealed that Gal-1 KO mice had a significantly lower percentage of macrophages found within the peritoneum compared to WT mice. A limitation of these values is that by reporting the populations as percentage it does not factor for the total number of leukocytes within the cavity of the mice. However, there are no reports to suggest that a difference in total leukocyte counts exists in naïve Gal-1 KO mice thus it could be postulated that this is an effect of efferocytosis and that these cells have already left the cavity having engulfed apoptotic cells. As previously discussed, it has been evidenced that Gal-1 KO macrophages phagocytose more effectively than WT cells when cultured *ex vivo* (Barrionuevo *et al.*, 2007), thus perhaps in this scenario they have done so and already departed the cavity. Furthermore, it has also been demonstrated that activated macrophages are the predominant source of endogenous Gal-1 (Rabinovich *et al.*, 1996, 1998) within the peritoneal cavity, thus perhaps they are retained in WT mice due to an altered phenotype. The difference in percentage of macrophages seen in this study was an unpredicted find, in line with the NC3Rs principles it was not considered essential for this assay to be repeated with an additional group of naïve WT and KO mice simply to determine the basal leukocyte counts within the peritoneal cavity, however if it could be incorporated into future work with the KO mice it would be useful for added perspective on the results obtained here.

The percentage of apoptotic neutrophils remaining in the cavity was also analysed and results were comparable between genotypes with an expected decline in percentage of free apoptotic neutrophils at 60 mins compared to 30 mins. In accordance with this

the percentage of macrophages positive for human neutrophils increased when assessed at 60 mins compared to 30 mins post injection of cells. Therefore, it is interesting that the decrease in percentage of macrophages in Gal-1 KO mice did not yield a significant effect on the subsequent percentage positive for ingesting apoptotic neutrophils. Indeed, it might be expected that if there was a reduction in macrophage numbers populating the peritoneum of Gal-1 KO mice that being challenged with an equal number of apoptotic neutrophils as WT mice (with their larger macrophage population) would result in an increased percentage of the macrophages having ingested neutrophils in Gal-1 KO mice, as a result of their increased ratio of neutrophils to macrophages. Likewise, there was no significant effect of genotype on relative number of apoptotic cells that had been engulfed per macrophage at either 30 mins or 60 mins, although arguably a subtle trend towards more apoptotic neutrophils ingested by Gal-1 KO macrophages was detected for the latter but this did not reach significance.

To reiterate what has been discussed earlier it is well established that following their ingestion of apoptotic neutrophils macrophages swiftly depart the inflammatory site consequently making efferocytosis a tricky process to capture *in vivo*. Certainly, this assay was a good attempt to establish whether the absence of Gal-1 has any significant effect on the ability of macrophages to recognise and ingest apoptotic neutrophils, a question which had not previously been addressed *in vivo*. None the less it is possible that some of the cells will have escaped analysis. Assays performed *ex vivo* have looked at phenotyping Gal-1 KO macrophages and have revealed significantly higher levels of MHC-II (Barrionuevo *et al.*, 2007) and a more ‘M2’ like phenotype, the latter demonstrated in this thesis by significantly higher CD206 and

lower CD86 than WT macrophages. Likewise, levels of the scavenger receptors CD36 and MerTK, showed a visible trend for increased expression on cells from Gal-1 null mice. These findings provide evidence in support of an enhanced phagocytic phenotype Gal-1 KO macrophages which may in turn suggest more efficient efferocytosis and subsequent departure from the site. Consequently, this may provide explanation for the lower macrophage percentage detected in Gal-1 KO mice in this experimental setting.

Regarding the production and release of endogenous Gal-1 by peritoneal macrophages in this assay it is important to reiterate that for this process to occur activation of macrophages is required. In this setting the naïve peritoneal cavity does not provide the required stimulation to activate these cells, indeed it is not until addition of apoptotic neutrophils into the system that such a signal is received. Thus, in this set up the secreted endogenous Gal-1 will not come into action until after the introduction of apoptotic neutrophils. Current literature reports that Gal-1 facilitates neutrophil clearance via a process termed “preaperesis” whereby neutrophils are efferocytosed without undergoing apoptosis (Dias-Baruffi *et al.*, 2003; Stowell *et al.*, 2007, 2009) via induction of PS exposure on the outer membrane of neutrophils independently of their apoptosis (Stowell *et al.*, 2009). However, the neutrophils in this assay are already apoptotic and consequently PS positive. As such, if endogenous Gal-1 is released by WT macrophages in response to their activation and induction of PS exposure is the sole role for the protein regarding clearance of neutrophils then its presence in this set up would appear redundant. It would be interesting to explore alternative ways in which the anti-inflammatory actions of Gal-1 might regulate efferocytosis, for example Gal-3 has been shown to function as an opsonin (Karlsson *et al.*, 2008), whether Gal-1

shares this behaviour is yet unreported in the literature however has been investigated as part of the scope of this thesis and will later be discussed in further detail (see section 4.6).

## *4.4.* Gal-1 Overrides Neutrophil Pro-Survival Factors and Induces Apoptosis.

### *4.4.1. Gal-1 Increases Phosphatidylserine Expression on Neutrophils.*

When viable neutrophils were assessed for surface PS exposure following short incubation periods with hrGal-1 significantly increased expression was detected on cells exposed to hrGal-1 *in vitro*. Contrary to the literature which reports that only activated and not resting neutrophils respond to exogenous Gal-1 by exposing PS on their surface (Stowell *et al.*, 2007), in this study it was shown that neutrophils freshly isolated from human peripheral blood had increased PS after just 1h with hrGal-1 in the absence of any additional stimulation and at much lower concentrations (1µM vs 10µM). This effect was also observed after only 30mins (data not included). It has been reported that induction of PS exposure by hrGal-1 is reversible and occurs in the absence of apoptosis (Stowell *et al.*, 2009), indeed the data reported in this current study is in line with this theory as the percent of PS positive cells was considerably higher than the percentage of the population likely to undergo spontaneous apoptosis within the short incubation period, particularly given the presence of serum in the media. The study by Stowell *et al.,* incubated cells with exogenous Gal-1 for 12h, assessed PS positivity and then used a lactose wash to remove exogenous Gal-1 bound to these cells, which were then incubated in complete media for 2 days and assessed again for PS exposure. The results showing reduced PS on this second assessment were used to deduce that the cells had not undergone apoptosis. However, their assay was performed on HL-60 cells, which although regarded as neutrophil-like, are a proliferating cell line and so the translatability of this finding to human neutrophils with a finite lifespan remains questionable. With regards to the unlikely scenario that the PS exposure seen in this current assay is the result of

neutrophils undergoing spontaneous apoptosis it could be postulated that Gal-1 is maintaining PS exposure as opposed to induction. Although a calcium dependent process, PS exposure has been associated with non-specific flip-flop of phospholipids (Bratton *et al.*, 1997), in this manner perhaps binding of Gal-1 to the cell surface is prohibiting the PS molecules 'flopping' back inwards. Indeed, it has been reported that galectin lattice formation following binding can effectively trap glycoprotein receptors at the cell surface (Rabinovich *et al.*, 2007), perhaps a similar action occurs as hrGal-1 binds to neutrophils, thus trapping the externalised PS. Likewise this explanation may provide rationale for the results from the study by Stowell *et al.* showing the reversal of PS exposure following removal of exogenous Gal-1 (Stowell *et al.*, 2009).

To investigate this phenomenon of early PS exposure further, neutrophils were also assessed after a 4h incubation. To mimic the scenario found in the rheumatoid joint, whereby mediators such as GM-CSF are known to prolong neutrophil lifespan the effect of Gal-1 was tested in combination with GM-CSF on PS exposure and/or early apoptosis of human neutrophils *in vitro.* Here it was shown that neutrophils treated with hrGal-1 still maintained increased phosphatidylserine exposure compared to unstimulated cells after a 4h incubation. Furthermore, the increased PS externalisation in response to hrGal-1 was maintained in the presence GM-CSF. As anticipated, PS exposure was notably less on cells with GM-CSF alone. In line with findings in the literature neutrophils incubated in low dose autologous serum are not yet PI+ positive at this early time point (Leitch *et al.*, 2010). Thus, in the absence of progression to a loss of membrane integrity it is difficult to determine whether these cells are early apoptotic or simply PS positive, particularly given that the latter marks the first step of the former. However, the existence of the GM-CSF pro-survival effect in this assay

suggests that after 4h neutrophils are beginning to undergo spontaneous apoptosis. The increased PS exposure in the presence of hrGal-1 alone at this time point supports the aforementioned theory of the externalised molecules being trapped and maintained on the external membrane. Neutrophils co-incubated with GM-CSF and hrGal-1 for 4h had significantly increased PS compared to GM-CSF only treated neutrophils and a notable increase compared to untreated cells, suggesting that exogenous Gal-1 has the dominant effect. Certainly the delay in neutrophil PS exposure linked with GM-CSF treatment was considerably less striking in the presence of hrGal-1, nonetheless its subtle effect compared to neutrophils incubated with hrGal-1 alone provides further support for commencement of apoptosis taking place at this time point.

In any case, analysis at a later time point was neccesary to determine if the actions of hrGal-1 on GM-CSF activated cells was simply an effect on PS exposure in the presence of exogenous Gal-1, as has been suggested (Stowell *et al.*, 2009), or whether exogenous Gal-1 influences the apoptotic process of neutrophils exposed to pro-survival factors, as has been demonstrated for neutrophils from the rheumatoid synovium (Cedeno-Laurent *et al.*, 2010). Thus, I examined the progression of neutrophils to late apoptosis following their extended incubation with or without GM-CSF and/or hrGal-1 by investigating morphology, mitochondrial membrane potential ($\Delta\Psi m$), and outer membrane integrity in addition to PS exposure to ascertain the effects of hrGal-1.

*4.4.2. Gal-1 Induces Apoptosis of Stimulated Neutrophils.*

Neutrophil survival and apoptosis are largely influenced by the microenvironment, and at sites of chronic inflammation the presence of pro-inflammatory mediators are effective at delaying the spontaneous apoptosis of neutrophils (Simon, 2003). To resemble the inflammatory environment of neutrophils within the rheumatoid joint, GM-CSF and the apolipoprotein SAA, both known to prolong neutrophil lifespan (Klein *et al.*, 2000; El Kebir *et al.*, 2007), were supplemented and the effect of hrGal-1 on the apoptosis of human neutrophils was investigated *in vitro.* Following overnight incubation in low serum, neutrophil apoptosis was primarily assessed by AnxV/PI staining and flow cytometry, cells that were positive for both AnxV and PI displayed externalised PS and membrane permeability respectively, indicative of cells in the late stage of apoptosis. In agreement with the literature, hrGal-1 did not alter the rate of spontaneous apoptosis of peripheral blood neutrophils (Stowell *et al.*, 2007), whilst GM-CSF significantly delayed this process (Klein *et al.*, 2000; Derouet *et al.*, 2004). HrGal-1 was able to partially reverse this effect of GM-CSF, suggesting, as was observed by Cedeno-Laurent *et al.* that in some environments exogenous Gal-1 facilitates induction of neutrophil apoptosis. Likewise, SAA also afforded neutrophils a notable degree of protection from their spontaneous apoptosis, with reversal of this evident following addition of hrGal-1. These results were supported by analysis of cell morphology and in line with the order of events following apoptosis, whereby nuclear condensation and cell shrinkage occur prior to loss of membrane integrity (Denecker *et al.*, 2000).

Besides PS exposure another early event as neutrophils undergo apoptosis is the disruption to mitochondrial membrane potential ($\Delta\Psi m$) (Kroemer *et al.*, 1998). Thus,

for verification of findings, neutrophils were also assessed by 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) staining, a cell-permeant fluorescent dye that is selective for the mitochondria of live cells. As expected, cells that were left to apoptose overnight had a reduced $DiOC_6$ signal indicative of reduced ΔΨm compared to those treated with GM-CSF. Interestingly, this method revealed discordant results for the effect of hrGal-1. Here it was shown that neutrophils undergoing apoptosis in the presence of hrGal-1 had increased ΔΨm, an effect which was concentration dependent with a positive correlation between concentration of hrGal-1 and ΔΨm. Furthermore, this was shown to be additive to the effect of GM-CSF, with neutrophils incubated with both GM-CSF and the highest concentration of hrGal-1 revealing the greatest ΔΨm. Commonly, disruption of the GM-CSF signalling pathway by immunosuppressive mediators induces a loss of ΔΨm in addition to PS exposure and nuclear changes (Woltman *et al.*, 2003), it is intriguing that hrGal-1 did not function in this way.

Existing literature on the influence of exogenous Gal-1 on ΔΨm is surprisingly limited, however findings from one study revealed that treatment with Gal-1 resulted in a significant increase in ΔΨm and an increase in cells with a hyperpolarised mitochondrial membrane (Matarrese *et al.*, 2005). When time-dependent changes in ΔΨm were assessed results showed that initially (6h-18h) an increased ΔΨm and hyperpolarization was shown in response to exogenous Gal-1 and that typical depolarization was a much later event (36h-72h) (Matarrese *et al.*, 2005). Although these findings are from assaying apoptosis in lymphocytes, it is possible that exogenous Gal-1 has the same effect on neutrophil ΔΨm. As such, it could be suggested that at the timepoint when ΔΨm was assessed by $DiOC_6$ in the current

study that cells treated with Gal-1 may still have been within the phase of hyperpolarization of the mitochondrial membrane, thus providing an explanation of the increased staining of $DiOC_6$. Furthermore, findings from another study have shown that Gal-1 induced apoptosis of cells independent of the activation of caspase-3 but instead involving the rapid nuclear translocation of endonuclease G (EndoG) from mitochondria (Hahn *et al.*, 2004). Here it was shown that EndoG translocation occurred prior to loss of ΔΨm, with an eventual loss of ΔΨm detected much later. Additionally, it was shown that Gal-1 did not activate caspases, nor was cell death blocked by inhibiting caspases indicating an alternative pathway (Hahn *et al.*, 2004). Again, this study was performed using lymphocytes but nonetheless strongly suggests that the actions of Gal-1 on apoptosis involves a unique pattern of mitochondrial events, an area which requires further investigation, particularly with regards to neutrophils.

Taken together the data obtained from the neutrophil apoptosis assays performed as part of this current study indicate that in the absence of activating stimuli (GM-CSF or SAA) hrGal-1 had no effect on cell viability. Importantly, this provided support for the existing finding that neutrophils from the synovial fluid of rheumatoid arthritis patients are susceptible to Gal-1 induced apoptosis (Cedeno-Laurent *et al.*, 2010). Indeed, an activated phenotype appears to render neutrophils susceptible to Gal-1-induced apoptosis. Curiously, an activated phenotype was not shown to be a requirement for modulating ΔΨm, in fact hrGal-1 was shown to augment ΔΨm in a manner seemingly independent of cell viability. Indeed, exogenous Gal-1 hyperpolarises the mitochondrial membrane and delays the loss of ΔΨm but it does not completely inhibit this process nor does this action influence cell survival (Matarrese *et al.*, 2005). This

would suggest that exogenous Gal-1 alters the pattern of mitochondrial events, a theory conflicting the current and long established understanding that reduction in ΔΨm in an essential early and irreversible step of programmed cell death (Zamzami *et al.*, 1995).

### *4.4.3. Gal-1 Delays GM-CSF Induced Phosphorylation of Signalling Pathways.*

Neutrophil apoptosis is controlled by an intricately connected and complex network of signalling pathways, including the ERK, AKT, and p38 MAPK pathways (Colotta *et al.*, 1992; Klein *et al.*, 2000; Khreiss *et al.*, 2002; József *et al.*, 2004). As part of this study these intracellular signalling pathways involved in neutrophil apoptosis were investigated by Western blot to determine the effect of hrGal-1 on neutrophils with or without stimulation from the pro-survival factor GM-CSF. In line with findings in the literature GM-CSF was shown to induce rapid phosphorylation of AKT within 1min (Klein *et al.*, 2000), with the same signalling response also evident for p38 MAPK and ERK phosphorylation. In line with reports in the literature, activation of neutrophil signalling by GM-CSF peaks early in the AKT and p38 MAPK pathways followed by a drop in phosphorylation at 15mins (McLeish *et al.*, 1998). Interestingly, when this signal began to decline in GM-CSF stimulated neutrophils, cells that had also been stimulated with hrGal-1 displayed an overall lessened depreciation from peak phosphorylation signal. At the earliest timepoint phosphorylation of AKT was notably higher in neutrophils stimulated with GM-CSF only, whereas later timepoints show hrGal-1 and GM-CSF co-treated neutrophils evidenced likewise phosphorylation to GM-CSF alone. Like AKT signalling at 1min, phosphorylation of ERK was also increased in neutrophils stimulated with GM-CSF only, however the inverse was

shown for neutrophils when stimulated for longer. Intriguingly, hrGal-1 co-stimulation with GM-CSF for 15mins resulted in increased phosphorylation of ERK, whether this signalling is potentiated or drops off in response to longer stimulation was not assessed as part of this study and thus remains of interest to investigate. Overall, this data suggests that hrGal-1 delayed GM-CSF induced activation of the AKT and ERK signalling pathways. Stimulating naïve neutrophils with hrGal-1 alone caused a modest signalling response with a slight increase in phosphorylation across sequential timepoints compared to unstimulated cells, however this was donor specific and requires further examination.

It is well established that tyrosine kinases are essential for the transduction of GM-CSF anti-apoptotic signals in neutrophils (Yousefi *et al.*, 1994; Vlahos *et al.*, 1995) and in line with existing findings results from this current study demonstrated GM-CSF induced tyrosine phosphorylation of intracellular proteins. Phosphoinositide 3-kinases (PI3-kinase) have been identified to have a significant intracellular role in controlling cell survival/death signalling with robust evidence existing for phosphorylation of AKT having a suppressive action on neutrophil apoptosis (Martínez *et al.*, 2006; Levett *et al.*, 2014). Mitogen-activated protein kinases (MAPKs) are another family of kinases involved with regulating cell viability. Activation of ERK has been primarily defined as inhibiting apoptosis, however the role for p38 MAPK remains more controversial (Xia *et al.*, 1995; Frasch *et al.*, 1998; Jarpe *et al.*, 1998). In general activation of p38 MAPK promotes cell death yet it has also been proposed to have anti-apoptotic functions upon phosphorylation (Klein *et al.*, 2000; Alvarado-Kristensson *et al.*, 2002). One explanation proposed for this is that apoptosis of neutrophils can either be stress-activated or spontaneous, the former reliant on activation of p38 MAPK the latter

independent of this requirement (Frasch *et al.*, 1998). An alternative theory to the presence of two distinct pathways leading to neutrophil apoptosis that may explain the dual effect of p38 MAPK is that phosphorylation of this MAPK does not guarantee its activation (Kumar *et al.*, 1999), indeed the downstream phosphorylation of the p38 MAPK specific substrate MAPK-activated protein kinase 2 (MK2) is required for p38 MAPK activity. It has been shown that GM-CSF induced phosphorylation of p38 MAPK in neutrophils is not suppressed by the pharmacological inhibitor of the p38 MAPK pathway SB203580 whereas the downstream phosphorylation of MK2 was (ten Hove *et al.*, 2007). Additionally treatment with SB203580 alone suppressed neutrophil apoptosis, which interestingly did not have an additive effect on survival in the co-presence of SAA (El Kebir *et al.*, 2007). These findings suggest that the pathway between p38 MAPK and MK2 is particularly susceptible to disruption, consequently it would be interesting to investigate this step of the apoptosis signalling cascade in GM-CSF stimulated neutrophils in response to hrGal-1.

However, in line with the data obtained in this study, it is worth noting that GM-CSF induced tyrosine-phosphorylation of p38 MAPK has been shown to be a comparatively weaker signal in neutrophils than other kinases (Suzuki *et al.*, 1999), whereas GM-CSF has been shown to activate both the PI3-kinase/AKT and ERK pathways in a number of cell types including robust activation in neutrophils (Tilton *et al.*, 1997; McLeish *et al.*, 1998). Likewise SAA has also been shown to induce a rapid, transient phosphorylation of AKT and ERK in freshly isolated neutrophils relative to unstimulated cells as well as enhanced phosphorylation of p38 MAPK (El Kebir *et al.*, 2007). It was shown that SAA suppression of neutrophil apoptosis is mediated via AKT and ERK signalling, which signal downstream targeting of BAD (El Kebir *et al.*, 2007).

Importantly, inhibiting the phosphorylation of either AKT or ERK kinase alone attenuated, though did not completely reverse, the apoptosis-delaying action of SAA (El Kebir *et al.*, 2007). Interestingly, it was also shown that inhibiting both kinases together did not produce an additive effect thus suggesting that AKT and ERK act in accordance with one another. Findings from the study by El Kebir *et al.,* also revealed that the aspirin triggered lipoxin 15-epi-$LXA_4$ can oppose SAA delayed neutrophil apoptosis and that SAA induced phosphorylation of ERK and AKT was attenuated by the lipoxin. Comparable findings in this current study may suggest that hrGal-1 is working by a similar mechanism of attenuating the initial phosphorylation to partially override the effect of GM-CSF on neutrophils.

Further investigation is required to determine the exact mechanism by which hrGal-1 is interrupting the pro-survival signal. From the findings of El Kebir *et al.* for 15-epi-$LXA_4$ on SAA stimulated neutrophils it is known that the opposing regulation of apoptosis occurs at the level of the receptor expression on the neutrophil surface, specifically through the formyl peptide receptor 2/lipoxin $A_4$ receptor to which both 15-epi-$LXA_4$ and SAA bind (Su *et al.*, 1999; He *et al.*, 2003; Chiang *et al.*, 2006; El Kebir *et al.*, 2008). Certainly, the pattern of tyrosine kinase phosphorylation is remarkably similar between the aforementioned study and the results obtained, with both GM-CSF and SAA phosphorylating all three of the kinases assayed, and an initially attenuated response with the addition of hrGal-1 and 15-epi-$LXA_4$ respectively. These kinases have a relatively early position with regards to the complete signalling cascade and their modulation suggests that hrGal-1 interferes with GM-CSF signalling further upstream than these molecules. Thus, it could be postulated that hrGal-1 exerts its effect at the level of the neutrophil cell surface.

Indeed, CD45 which is a member of the tyrosine phosphatase family and is known to dephosphorylate Src kinases involved in the GM-CSF signal transduction pathway is also a known receptor for Gal-1 on the surface of neutrophils. Specifically, CD45 regulates the Src kinase family member Lyn by reducing phosphorylation of the tyrosine inhibitory site (Y507) with dephosphorylation at this site resulting in activation of Lyn (Young *et al.*, 2003). On the other hand, phosphorylation of Lyn at this site correlates with inefficient signalling through the GM-CSF receptor (Wei *et al.*, 1996; Hibbs and Harder, 2006; Scapini *et al.*, 2009). Consistent with this finding a study by Wei et al., identified Lyn as a critical tyrosine kinase responsible for transducing GM-CSF survival signals in neutrophils. Results revealed that Lyn is physically coupled to the intracellular portion of the beta subunit of the GM-CSF receptor and that only the antisense of Lyn and not any other Src family member could reverse the GM-CSF stimulated pro-survival effect (Wei *et al.*, 1996). Importantly binding of Gal-1 to CD45 inhibits its protein phosphatase activity and therefore the dephosphorylation of Lyn kinase (Fouillit *et al.*, 2000). The identification of two tyrosine phosphorylation sites on Lyn kinase infers the complexity of regulation of its signalling, the autophosphorylation site is stimulatory while the C-terminal phosphorylation site has been indicated to be inhibitory, with the former constitutively phosphorylated in untreated cells and the latter dominant for its activity (Yanagi *et al.*, 1996). In this manner, CD45 regulates activity of Lyn by maintaining an unphosphorylated state at the C-terminal site, whereas the presence of Gal-1 results in phosphorylation of this site (Fouillit *et al.*, 2000). Furthermore, downstream of Lyn sits the PI3 Kinase/AKT pathway, whereby increased Lyn signalling has been demonstrated to enhance activation of AKT (Iqbal *et al.*, 2010).

Regarding the hrGal-1 mediated reversal of SAA pro-survival signals (see section 4.4.2) reported as part of this thesis, there are currently no published studies on SAA or its receptor on neutrophil FPR2 being linked to activation of the Lyn signalling pathway. Interestingly it has been reported that Lyn is coupled to the intracellular domain of CD36 in a similar manner to which it is paired with the GM-CSF receptor on neutrophils (Baranova *et al.*, 2010). Importantly, CD36, which acts as a scavenger receptor and is expressed on the surface of macrophages, is also a known SAA receptor on these cells, furthermore SAA binding to CD36 is suggested to induce activation of Lyn (Baranova *et al.*, 2010). An additional study has also shown that exposure of microglia to amyloid peptides results in activation of Lyn (McDonald *et al.*, 1997). However it has not been reported that Gal-1 interacts with these receptors, as such it may be more feasible in the case of SAA that two separate pathways are being initiated, SAA binding to FPR2 inducing pro-survival and hrGal-1 binding to CD45 inhibiting the dephosphorylation and subsequent activation of Lyn, with AKT downstream of both pathways. To establish whether this hypothesis or any of the theories put forward are correct further investigation of the signalling pathways which may be harnessed as the mechanism of action for hrGal-1 are required.

### *4.4.4. Potential Role for AnxA1 in Mediating Gal-1 Reversal of Pro-Survival.*

Annexin A1 (AnxA1), an endogenous anti-inflammatory protein, contributes to the resolution of inflammation through various ways, including dampening neutrophil recruitment, overriding pro-survival signals and promoting clearance (Perretti *et al.*, 1996; Solito *et al.*, 2003; Damazo *et al.*, 2006; Vago *et al.*, 2012), as well as increasing monocyte recruitment and efferocytosis (Arur *et al.*, 2003; Scannell *et al.*, 2007;

McArthur *et al.*, 2015). It is well established that neutrophils contain high levels of AnxA1 in their cytoplasm and granules and that upon cell stimulation AnxA1 is secreted (Perretti *et al.*, 1996; Perretti and D'Acquisto, 2009). Particularly relevant to this study is the finding that AnxA1 is released from neutrophils as they undergo apoptosis (Arur *et al.*, 2003; Scannell *et al.*, 2007). Furthermore, neutrophil AnxA1 expression has been shown to decrease in response to GM-CSF stimulation, a finding which may suggest its secretion (Langereis *et al.*, 2011). As such this was investigated as part of the current study to establish whether modulation of AnxA1 expression may be a mechanism utilised by hrGal-1 to exert its anti-inflammatory actions.

Neutrophils demonstrated a trend for the reduction of surface AnxA1 after 4h and sequentially at 20h compared to levels expressed on freshly isolated cells, with no significant modulation resulting from stimulation with GM-CSF and/or hrGal-1. However, it is worth noting that following 4h incubation surface AnxA1 was lower on neutrophils incubated with GM-CSF compared to control cells, a trend which although subtle was shown to reverse at the 20h timepoint. Relative to total expression, surface levels of AnxA1 are low indicating that the protein is not maintained on the neutrophil surface and suggesting that its detection here may be the result of capturing the protein as it is being secreted through the neutrophil membrane. Indeed, translocation of AnxA1 to the cell membrane has been observed upon cell activation (Perretti *et al.*, 1996).

It is well established that AnxA1 is predominantly intracellular; in resting neutrophils, monocytes and macrophages high levels of cytoplasmic AnxA1 have been detected (Morand *et al.*, 1995; Perretti *et al.*, 2000). In this current study the total AnxA1 levels

of unstimulated neutrophils was revealed to increase with time, with the highest expression of the protein detected at 20h, suggesting that synthesis of the protein is increased in neutrophils as they undergo apoptosis. In line with this suggestion endogenous AnxA1 release from apoptotic neutrophils has been reported (Scannell *et al.*, 2007). The findings presented as part of this thesis show a trend towards a reduction in total AnxA1 levels for neutrophils stimulated with GM-CSF, which increases over time. Interestingly, data for apoptotic neutrophils at 20h reveals the GM-CSF induced reduction of AnxA1 was partially reversed by the co-incubation of cells with hrGal-1. However, there appeared to be no effect of hrGal-1 alone. One theory which may explain these results is that the expression of AnxA1 increases as neutrophils undergo apoptosis, as the 0h, 4h and 20h data here suggests, and consequently the GM-CSF induced rescue from apoptosis of neutrophils directly results in, or from, a reduction in AnxA1 expression. Alternatively, it could be suggested that GM-CSF stimulation of neutrophils did not modulate expression levels but caused increased secretion, as previously suggested (Langereis *et al.*, 2011). To attempt to establish which of these hypotheses likely explains the modulation of AnxA1 expression in response to GM-CSF, and thus determine the actions of hrGal-1 in this setting, neutrophil release of AnxA1 was quantified.

It was shown that GM-CSF reduced neutrophil release of AnxA1, an effect that was particularly apparent in apoptotic neutrophils at 20h and shown to be markedly reversed in neutrophils co-incubated with hrGal-1. Taken together these findings lend support to the former argument that GM-CSF stimulated neutrophils express and release less AnxA1 and that this is most likely a direct result of their increased viability. In line with this suggestion the opposite effect has been reported for pro-apoptotic

stimuli whereby induction of AnxA1 expression and externalization was shown to be coupled with the proapoptotic effect of a histone deacetylase inhibitor (FK228) in leukaemia cells (Tabe *et al.*, 2007).

Certainly, in the context of inflammation it could easily be argued that GM-CSF reduces the release of anti-inflammatory AnxA1 by neutrophils removing an endogenous braking system from an already self-perpetuating inflammatory response, the explanation for this being three-fold by affecting recruitment, survival and clearance. Firstly, the externalised form of endogenous AnxA1 has been shown to inhibit trans-endothelial migration of neutrophils consequently abating their infiltration to the inflammatory site (Perretti *et al.*, 1996; Gastardelo *et al.*, 2009; Pederzoli-Ribeil *et al.*, 2010). Secondly, endogenous AnxA1 has an important pro-apoptotic function and blocking this pathway abolishes both morphological and biochemical changes associated with apoptosis (Vago *et al.*, 2012). Thirdly, following neutrophil release of endogenous AnxA1 it colocalises with PS on the apoptotic cell surface and promotes efferocytosis (Arur *et al.*, 2003; Scannell *et al.*, 2007). As such the ability of hrGal-1 to reverse the effect of GM-CSF on reducing AnxA1 expression may be a previously undiscovered mechanism by which Gal-1 overrides pro-survival signals in neutrophils. Furthermore, as well as the increase of AnxA1 resulting from the addition of hrGal-1 to GM-CSF treated neutrophils there is also increased expression of PS on these cells, (as previously discussed in sections 4.4.1 and 4.4.2). Indeed, the correlation of these two results is notable due to the colocalization of PS and AnxA1 being important for facilitating macrophage efferocytosis of neutrophils (Arur *et al.*, 2003).

### *4.4.5. Summary.*

In contempt of the fact that externalised PS is conventionally associated with apoptosis the data presented here indicates that the effect of hrGal-1 on PS exposure and its ability to override neutrophil pro-survival signals to induce apoptosis are distinct and unconnected actions. It is known that hrGal-1 binding to viable neutrophils results in enhanced expression of PS on the extracellular membrane. However, in disagreement with previous reports suggesting that exogenous Gal-1 'induces' PS exposure it is perhaps more perceivable that it simply retains the molecules on the outer membrane. The phospholipid membrane has a degree of fluidity and consequently non-specific flip-flop of phospholipids can occur with no net impact on membrane integrity. Furthermore, the binding and subsequent crosslinking of galectins at the cell surface has been shown to maintain receptor externalisation/expression. This theory would also fit with the findings of PS exposure in the presence of other galectins and its subsequent reversal on galectin removal (Stowell *et al.*, 2007, 2009). Additionally it has also been suggested that galectins need to be in their dimerized form to have an action on PS exposure, which likewise has been shown to be required for efficient galectin receptor cross-linking and lattice formation at the cell surface (Demetriou *et al.*, 2001; Brewer *et al.*, 2002; Nieminen *et al.*, 2007).

It has been put forward that PS exposure in the absence of apoptosis may function in the resolution of inflammation to aid efferocytosis, importantly not just of apoptotic cells but also of healthy neutrophils present at the inflammatory site. This process whereby neutrophils are efferocytosed without undergoing apoptosis has been termed "preaperesis" (Dias-Baruffi *et al.*, 2003; Stowell *et al.*, 2007, 2009), however it is yet to be definitively proven as a mechanism of clearance. Indeed, phospholipid exposure

has been reported to provide 'find-me', 'eat me' and 'tolerate me' signals to phagocytes and typically considered an eat me signal PS exposure can also act as a 'tolerate me' signal to stimulate release of anti-inflammatory cytokines (Biermann *et al.*, 2013). As a result of membrane remodelling PS has a higher lateral mobility on the surface of apoptotic cells, thus phagocytes are able to distinguish between living and apoptotic cells, with specific PS distribution required to trigger the correct receptor clustering on the phagocyte to facilitate efferocytosis (Biermann *et al.*, 2013). These findings would imply that the concept of ‘preaperesis’ may not be a successful mechanism for clearance of non-apoptotic neutrophils. Perhaps, galectin binding to the surface of neutrophils is instead a strategy to hold PS on the outer membrane, promoting its exposure to macrophages to stimulate their release of anti-inflammatory cytokines. This would suggest an important pro-resolving behaviour for Gal-1 in triggering the premature release of anti-inflammatory cytokines which would ordinarily be dependent on PS exposure by neutrophils as they initiate apoptosis. In line with this theory the increased PS expression on hrGal-1 treated cells started to plateau at 4h compared to 1h and appeared to be completely ablated by 20h, having no effect on progression of apoptosis. Certainly, unstimulated neutrophils begin to undergo spontaneous apoptosis thus their natural PS exposure negates the requirement for involuntary expression.

In addition to its initial effect, hrGal-1 also has a later purpose and mediates reversal of delayed neutrophil apoptosis. Findings from this study have confirmed the ability of hrGal-1 to override pro-survival signals and induce apoptosis of neutrophils. This has been indicated by changes in cell morphology and outer membrane integrity in addition to PS exposure. Importantly hrGal-1 was shown to modulate the intracellular signalling

pathways involved in controlling cell survival as well as modulate the expression and subsequent release of endogenous AnxA1, an action associated with neutrophil apoptosis and endowed with anti-inflammatory functions. It is well established that delayed neutrophil apoptosis facilitates many acute and chronic inflammatory diseases and appears to be largely mediated by excessive production of GM-CSF (Dibbert *et al.*, 1999; Saba *et al.*, 2002). However, the activated phenotype of these neutrophils appears to render them susceptible to Gal-1-induced apoptosis, a discovery first suggested from a study on neutrophils from the synovial fluid of rheumatoid arthritis patients (Cedeno-Laurent *et al.*, 2010). The ability of hrGal-1 to reverse the effects of GM-CSF (and other neutrophil pro-survival factors) is a promising finding and indicates the potential therapeutic application of Gal-1 in inflammatory diseases whereby the accumulation of chronically activated neutrophils stimulates an unrelenting and often self-perpetuating immune response.

### *4.5. Gal-1 Binds to a Receptor on Neutrophils that is Upregulated by GM-CSF.*

#### *4.5.1. Gal-1 Binds Viable but not Apoptotic Neutrophils.*

It is well established and has been demonstrated within this study, that exogenous Gal-1 can bind to the surface of viable neutrophils and that this occurs through a carbohydrate dependent interaction (Cooper *et al.*, 2008). However, there are conflicting suggestions as to whether Gal-1 binding to neutrophils occurs more readily when cells are of an activated phenotype. In an assay where freshly isolated neutrophils were incubated with Gal-1 in the presence or absence of platelet activating factor (PAF), there was no notable increase in Gal-1 binding to the activated cells (Cooper *et al.*, 2008). In an alternative study neutrophils that were stimulated with N-Formyl-Methionyl-Leucyl-Phenylalanine (fMLP) exhibited increased Gal-1 binding compared to unstimulated resting cells (Dias-Baruffi *et al.*, 2003) interestingly the latter study also revealed that exogenous Gal-1 binding was most abundant on enzymatically desialylated resting neutrophils, a finding which in addition to lactose inclusion inhibiting Gal-1 binding (even to activated cells) provided further verification of the carbohydrate dependent nature of the binding. Another important consideration is that mobilisation of different neutrophil granules is also stimuli specific and results in modulation of cell surface receptor expression (Borregaard *et al.*, 1994; Bratt *et al.*, 1999). Taken together these findings indicate that the impact of neutrophil activation on Gal-1 binding is stimuli dependent and more directly linked to differentially modulating the glycophenotype (expression of glycolipids and glycoproteins) on the neutrophil surface.

The effect of stimulation with GM-CSF and apoptosis of neutrophils on surface Gal-1 has not previously been reported, as such it was examined as an aspect of this study. Here it was shown that in the absence of stimuli apoptotic neutrophils had similar levels of endogenous Gal-1 on their surface as viable cells, a finding that indicates that Gal-1 surface expression is not modulated on naive neutrophils as they undergo apoptosis, and also implies that in the absence of stimuli intracellular Gal-1 is not externalised. Strikingly, the addition of GM-CSF to this assay resulted in increased endogenous Gal-1 being detected on the neutrophil surface. In line with this finding Gal-1 has previously been detected in the cytoplasm, nucleus and outer plasma membrane of activated neutrophils (Kurihara *et al.*, 2010) despite not being constitutively expressed in resting neutrophils (Dias-Baruffi *et al.*, 2010).

Neutrophils that had undergone apoptosis in the presence of hrGal-1 showed increased Gal-1 on their surface compared to naïve apoptotic neutrophils. Although it is unsurprising that bound Gal-1 was detected on the cell surface it is interesting that less exogenous Gal-1 bound to the surface of apoptotic cells than viable neutrophils, despite the considerably longer incubation period. This might suggest that no additional binding of Gal-1 occurs as neutrophils progress through apoptosis and/or that already bound Gal-1 dissociates from the surface of neutrophils as they undergo this process. In either case these results would suggest that changes to the neutrophil glycophenotype take place in parallel with apoptosis. Indeed, data obtained from lectin binding assays performed in collaboration with another member of the lab detected notable modulations between viable and apoptotic cells, yet there appeared to no consistent effect of GM-CSF and/or hrGal-1. However further investigation is required to verify these preliminary results. To address whether it was likely that Gal-1 easily

dissociates from the surface of apoptotic neutrophils lactose was added, but the lack of effect indicates that Gal-1 binding to the neutrophil surface is not easily disrupted.

It is also feasible that expression of the Gal-1 receptor(s) on neutrophils is modulated during the apoptotic process. Analysis of apoptotic neutrophils revealed two distinct subpopulations, Gal-1 high and Gal-1 low. The Gal-1 high population was detected irrespective of whether the exogenous protein had been added to the neutrophils indicating that the increased expression of Gal-1 likely represented mobilisation of the endogenous protein. To establish whether the cell surface of apoptotic neutrophils was modified in a manner to modulate Gal-1 receptor expression and effect the ability of the exogenous protein to bind, apoptotic cells were incubated with hrGal-1. The detected increase in Gal-1 was suggestive of exogenous Gal-1 having bound to the surface of the apoptotic neutrophil population. However, regardless of the addition of hrGal-1, there were two distinct subpopulations, Gal-1 high and Gal-1 low. The Gal-1 high population was comparable in hrGal-1 treated to control neutrophils, with no change in Gal-1 expression in response to hrGal-1 thus indicating that it did not bind the Gal-1 high subpopulation. Conversely the Gal-1 low population demonstrated a clear shift in the histogram peak, here increased Gal-1 was detected following incubation with hrGal-1, suggesting that the exogenous protein had bound to the Gal-1 low population. Importantly, when Gal-1 high and Gal-1 low subpopulations were assessed by size and granularity it was apparent that the Gal-1 high neutrophils with their decreased size and granularity were late apoptotic whereas the Gal-1 low population formed a tight cloud of larger and more granular cells indicative of viable or early apoptotic neutrophils.

Taken together these findings support the earlier suggestion that hrGal-1 freely and rapidly binds to the surface of viable neutrophils and that this can be differentially modulated on activated cells, yet it does not bind to the surface of neutrophils undergoing apoptosis. Importantly, in the context of neutrophils present at the inflammatory site the existence of nearby apoptotic cells and their released mediators does not impede hrGal-1 binding the remaining viable cells, a significant virtue further illustrating its therapeutic potential.

### *4.5.2. Gal-1 Receptor CD45 is Upregulated on GM-CSF Stimulated Neutrophils.*

Members of the galectin family are carbohydrate binding proteins with a general specificity for β-galactosides. Each galectin exhibits subtle preferences in oligosaccharide-binding specificity with Gal-1 known to favour poly-*N*-acetyllactosame (poly-LacNAc) chains (Caron *et al.*, 1990). As such, glycoconjugate ligands for Gal-1 include the extracellular matrix proteins laminin and fibronectin, as well as the serum glycoprotein Mac-2 binding protein (90K/Mac-2BP) and the cell surface receptors CD45, CD43, CD7, CD2, CD3 and GM1 (reviewed in (Rabinovich *et al.*, 2002)).

CD43 and CD45 are the main Gal-1 specific transmembrane glycoproteins highly expressed on neutrophils. Expression of these receptors was assessed following incubation with GM-CSF to determine if this was modified by stimulation with the pro-survival protein. CD45, but not CD43, was markedly upregulated on the neutrophil surface in response to GM-CSF. In line with this finding a previous study has shown that activated neutrophils exhibit increased expression of Gal-1 receptors (Almkvist *et al.*, 2002). Furthermore, within the same study it was proposed that neutrophil granule mobilisation is the mechanism behind this. It is well established that activation of

neutrophils results in mobilisation of their intracellular granules, and it has previously been demonstrated that the mobilization of receptor-storing granules and their subsequent fusion with the neutrophil plasma membrane results in increased receptor expression on the cell surface (Sengeløv *et al.*, 1995). Indeed, studies have shown that GM-CSF induces neutrophil granule exocytosis and receptor expression (Smith *et al.*, 1990; Graves *et al.*, 1992). In a study by Almkvist *et al.* it was suggested that Gal-1-binding epitopes that are not already expressed on the neutrophil surface are stored elsewhere in the cell. Additionally the evidence that CD45 antigens are involved in the regulation of stimulation by GM-CSF (Broxmeyer *et al.*, 1991; Suh *et al.*, 2005), provides further rationale that CD45 receptor mobilisation occurs in neutrophils activated by GM-CSF stimulation. Likewise, from the perspective of the intracellular signalling response if the Src family kinase Lyn is governing the pathway, then GM-CSF requires the tyrosine phosphatase CD45 to regulate activity of Lyn by maintaining an unphosphorylated state at the active C-terminal site for the pro-survival signal to proceed. Therefore, increasing expression of CD45 is advantageous for perpetuating the GM-CSF induced signalling cascade.

Detection of CD45 using an anti-CD45 antibody was compromised by hrGal-1 in a concentration dependent response. The affinity of Gal-1 binding to CD45 shown here was not unexpected given that it is such a heavily glycosylated protein. Alternative splicing has resulted in the generation of multiple CD45 isoforms (Thomas, 1989), yet analysis of the sugar chains has revealed that these isoforms ubiquitously contain large amounts of poly(N-acetyllactosamine) (Sato *et al.*, 1993; Furukawa *et al.*, 1998), to which Gal-1 will bind. Incubation with increased concentrations of hrGal-1 reduced anti-CD45 binding to the neutrophil irrespective of whether GM-CSF was included in the assay, suggesting that hrGal-1 binding prohibits the antibody binding to its antigen.

In line with this finding, it has been shown in a study on B cells that an antibody to CD45 was able to dissociate Gal-1 (Fouillit *et al.*, 2000). Furthermore, the binding of exogenous ligands to CD45 has been shown to result in CD45 dimerization (Weiss and Schlessinger, 1998). Importantly, ligand induced dimerization of CD45 has been suggested to block the catalytic site thus preventing the required substrate binding necessary for phosphatase activity (Weiss and Schlessinger, 1998). In this manner CD45 function may be negatively regulated by hrGal-1 binding.

Conversely very little modulation was detected for CD43 expression in this assay. In opposition to CD45 the glycoprotein CD43 is rapidly shed from the neutrophil membrane upon activation (Rieu *et al.*, 1992; Mambole *et al.*, 2008). Thus, the decrease in CD43 expression that was observed in response to GM-CSF in this present study was not unexpected. Here, in line with findings in the literature, the effect of GM-CSF stimulation on neutrophil CD43 membrane expression was only very moderate (Rieu *et al.*, 1992). Interestingly, existing evidence suggests that CD43 has an important role in neutrophil trafficking and generally serves as an anti-adhesive molecule to attenuate neutrophil-endothelial interactions (Matsumoto *et al.*, 2008). Thus, it is plausible that a ligand-receptor binding interplay between Gal-1 and CD43 may play an important role in facilitating the inhibitory effect of Gal-1 on neutrophil recruitment. Furthermore, CD43 has also been demonstrated to serve as a ligand and provide pro-adhesive actions on neutrophil recruitment, a behaviour which is context dependent (Matsumoto *et al.*, 2008), but may explain the divergence in the effect of endogenous Gal-1 seen in the *in vivo* models performed as part of this study.

*4.5.3. Summary.*

In this assay it was shown that Gal-1 does not bind to late apoptotic neutrophils. It could be suggested that this is due to changes in the glycophenotype that result from the rearrangement of phospholipids and loss of membrane integrity of these cells. Due to the loss of CD43 in response to activation of neutrophils it is possible that this receptor is also shed as neutrophils undergo apoptosis, although this hypothesis is yet to be investigated. Regarding the loss of Gal-1 binding to the neutrophil surface via CD45 it could be suggested that changes to the extracellular membrane during apoptosis results in either rearrangement or masking of the required glycan epitopes. It could be postulated that CD45 undergoes dimerization during neutrophil apoptosis, which would explain not only the loss of ligand binding potential but also the loss of tyrosine phosphatase activity.

Taken together these findings suggest that hrGal-1 binds only to viable neutrophils and that this occurs predominantly via CD45 expressed on the outer membrane of the cell. The activation of neutrophils by GM-CSF stimulation mobilises neutrophil intracellular granules to migrate to the outer membrane. Once positioned, these granules bind to the membrane allowing the release of their contents. Previous findings and results from this present study suggest that intracellularly contained Gal-1 binding epitopes, such as CD45 and low levels of endogenous Gal-1 may be exposed at the cell surface in this manner. Here the increased expression of CD45 provides for enhanced hrGal-1 binding and crosslinking resulting in ligand induced receptor dimerization of CD45. As a consequence of this change in formation the catalytic site of CD45 is masked, thus inhibiting substrate binding and the tyrosine phosphatase activity of CD45. By this mechanism the Src family kinase Lyn, which is

coupled to a GM-CSF receptor subunit, ceases to remain dephosphorylated at its inhibitory C-terminal site. Phosphorylation at this site renders the kinase inactive and thus signalling to the downstream PI3K/AKT pathway is discontinued and the GM-CSF induced pro-survival signal desists (figure 64).

Indeed, this mechanism is suggestive of a promising therapeutic application of exogenous Gal-1 for targeting GM-CSF stimulated neutrophils and inducing their apoptosis. Although the ligand receptor binding for Gal-1 and CD45 have been investigated in this study further work is required to assess the potency of this interaction to establish if it would still have effect in an inflammatory environment where GM-CSF production is sustained. The pro-survival effect of GM-CSF requires ongoing ligand-receptor interaction however due to neutrophils utilisation of GM-CSF exogenous GM-CSF is depleted (De Alessandris *et al.*, 2019). These findings may suggest in the *in vitro* assay presented in the current study that the availability of the growth factor is depleted over time. As GM-CSF levels within the culture media were not measured it is unknown whether this would be the case, but a high concentration was used (50ng/ml) so this seems unlikely. However, it does raise the question whether in a chronically inflamed environment (with excess GM-CSF) Gal-1 would still be capable of overriding the growth factor to induce neutrophil apoptosis. In this context GM-CSF ligand to receptor binding actions may need to be investigated and additionally targeted in order to maximise the efficacy of Gal-1 and promote the resolution of inflammation.

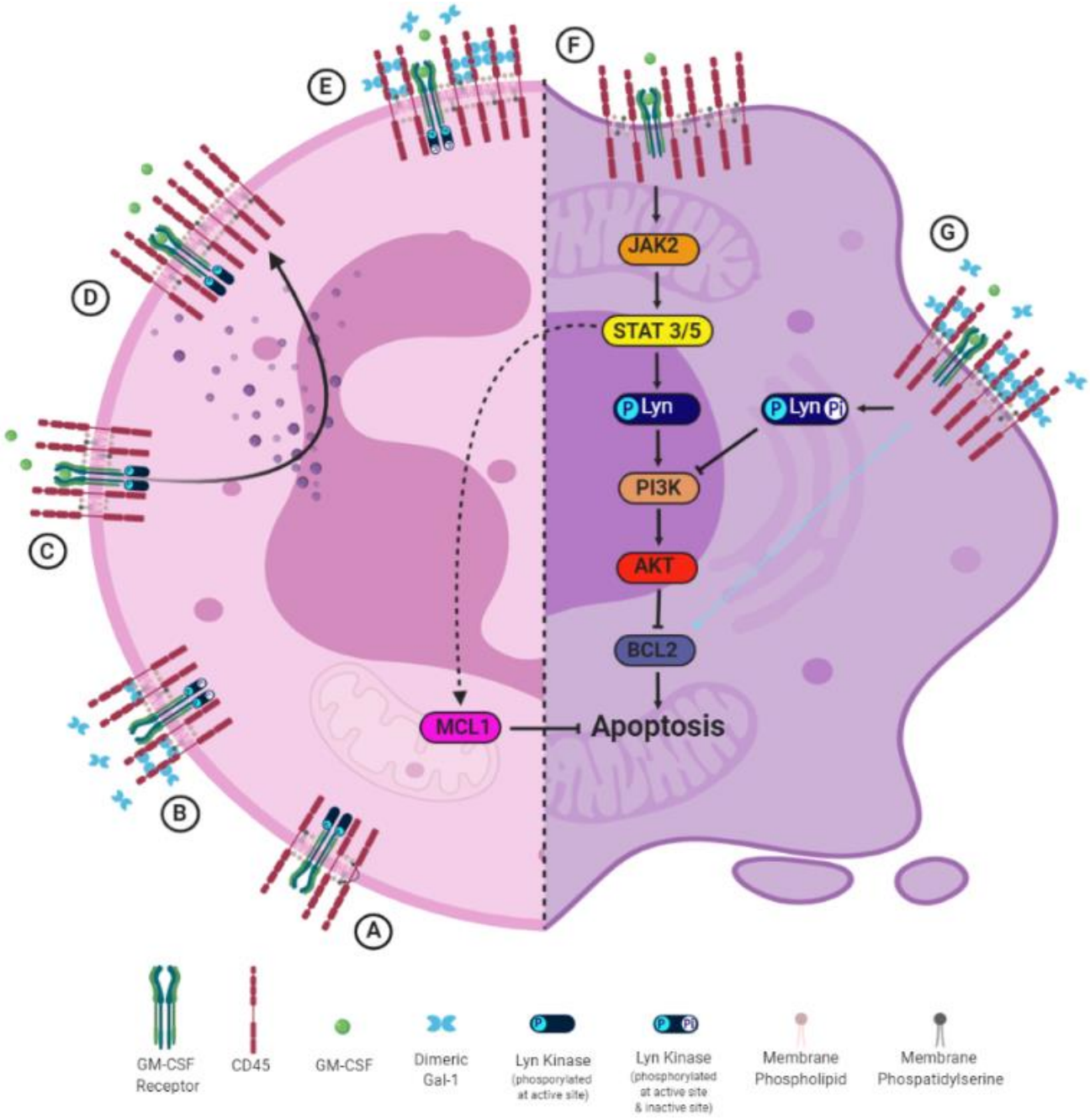



**Figure 64. Proposed signalling pathway of Gal-1 reversal of GM-CSF signalling.**
(**A**) Lyn kinase is coupled to the intracellular portion of the β subunit of the GM-CSF receptor. CD45 is a tyrosine phosphatase present on the neutrophil membrane that maintains Lyn activity by dephosphorylating the inhibitory site (Pi). (**B**) The binding of Gal-1 to CD45 results in CD45 dimerization and galectin lattice formation, blocking phosphatase activity at the inhibitory site (Pi) on Lyn and trapping receptors and exposed PS on the outer membrane. (**C**) GM-CSF requires CD45 to regulate activity of Lyn by maintaining an unphosphorylated state at the inhibitory site (Pi) for the pro-survival signal to proceed. (**D**) GM-CSF stimulates mobilization of receptor storing granules and their subsequent fusion with the plasma membrane, resulting in increased CD45 receptor expression on the cell surface to perpetuate the GM-CSF induced signalling cascade. (**E**) The presence of Gal-1 competes with the pro-survival signals of GM-CSF by inhibiting Lyn activity. (**F**) Downstream of Lyn sits the PI3 Kinase/AKT pathway. GM-CSF and increased Lyn signalling induce phosphorylation of AKT which delays apoptosis via inhibition of BCL2 activity. (**G**) Gal-1 binding to CD45 inhibits phosphatase activity. Lyn becomes phosphorylated at the inhibitory site, rendering the kinase inactive and preventing the anti-apoptotic signalling pathway being followed. [Created using BioRender illustrating tool.]

### *4.6. Efferocytosis was not Influenced by Gal-1 Treatment of Neutrophils.*

Findings in the literature have predominantly focused on the effect of exogenous Gal-1 on macrophages or on neutrophils alone, with studies on the process of their efferocytosis being particularly limited. One study which has investigated efferocytosis was tailored to the effect of exogenous Gal-1 on macrophages in this setting. Here it was revealed that Gal-1 promotes an anti-inflammatory pro-resolving ($CD11b^{low}$) phenotype in macrophages resulting in their efferocytic satiation being reached more quickly (Rostoker *et al.*, 2013). Likewise studies which have not assessed efferocytosis but have investigated the effect of Gal-1 on macrophages also indicate a pro-resolving phenotype, revealing that Gal-1 treated macrophages favour the alternative activation pathway, have a more M2 like phenotype, have reduced MHC-II expression and release lower amounts of pro-inflammatory mediators (Rabinovich *et al.*, 2000; Correa *et al.*, 2003; Barrionuevo *et al.*, 2007; Kogawa *et al.*, 2011; Abebayehu *et al.*, 2017). Regarding the role of neutrophils in efferocytosis a number of studies exist suggesting exogenous Gal-1 induces PS exposure alongside the hypothesis that this facilitates preaperesis, a process whereby these PS exposing neutrophils are efferocytosed in the absence of their apoptosis (Dias-Baruffi *et al.*, 2003; Stowell *et al.*, 2007, 2009). However, this line of research has yet to be extended to the clearance of these neutrophils and therefore was addressed as part of the scope of this thesis. Due to the previously highlighted difficulties of capturing efferocytosis *in vivo* these assays were performed *in vitro* using human cells to determine if hrGal-1 modulates this process.

In the present study efferocytosis assays were performed with neutrophils that had been exposed to hrGal-1. No difference was detected in the phagocytic uptake of neutrophils by macrophages as a result of cells undergoing apoptosis ± hrGal-1 ± GM-CSF. As previously discussed, assessment of the level of neutrophil apoptosis in response to GM-CSF and/or hrGal-1 revealed an increased percentage of viable cells in the GM-CSF treated population. However, this did not appear to have an impact on their efferocytosis, a finding which is likely explained by most of these neutrophils being PS positive and apoptotic, thus there was not enough difference in the viable percentage to confer a difference in efferocytosis. The lack of effect of hrGal-1 in this experimental setting is not altogether unexpected as the percentage of PS positive cells was not different between the hrGal-1 and hrGal-1 + GM-CSF groups when compared to unstimulated neutrophils. Previous assays have determined that unstimulated neutrophils have lower surface Gal-1 expression compared to stimulated cells. Yet, in this setting the level of surface Gal-1 expression on the neutrophil was not shown to influence their phagocytic uptake by macrophages, a finding which will be further discussed later.

Reports in the literature suggest that Gal-1 promotes efferocytic satiation of macrophages (Rostoker *et al.*, 2013), an effect that was established to be the result of Gal-1 acting directly on the macrophages. However, in the study by Rostoker *et al.* Gal-1 was administered into the peritoneal cavity during the resolving phase of peritonitis, therefore it had the potential to also modulate neutrophil phenotype and numbers. To further validate that the *in vivo* data was neutrophil-independent the *in vitro* set up performed as part of this present study was assayed at two different macrophages to apoptotic neutrophil ratios. As expected, a higher neutrophil to

macrophage ratio resulted in increased efferocytosis. Furthermore, the effect of neutrophils undergoing apoptosis ± hrGal-1 ± GM-CSF had no bearing on efferocytic uptake regardless of the number of apoptotic neutrophils overlaid. Thus, supporting that the effect evidenced in the *in vivo* model by Rostoker *et al.* was macrophage mediated.

Interestingly, Gal-3 has been reported to function as an opsonin (Karlsson *et al.*, 2008), to determine whether Gal-1 could behave in a mechanistically similar way both viable and apoptotic neutrophils were incubated with hrGal-1 immediately prior to and during their addition to macrophages. In these assays hrGal-1 did not modulate efferocytosis of neutrophils therefore establishing that, unlike Gal-3, Gal-1 was not shown to act as an opsonin.

Furthermore, this experimental set up provided an opportunity to observe the effect of hrGal-1 induced PS exposure on non-apoptotic neutrophils in the context of efferocytosis. Here, viable neutrophils incubated for 1h with hrGal-1 had significantly increased PS displayed on their outer membrane compared to naïve cells and by overlaying them on macrophages it was possible to investigate the theory of 'preaperesis' (Dias-Baruffi *et al.*, 2003; Stowell *et al.*, 2007, 2009). In this assay PS exposure was not shown to increase macrophage engulfment of these cells, thus suggesting that PS exposure in the absence of apoptosis did not provide the appropriate 'eat me' signal to macrophages and is not a mechanism for cell clearance. This finding is supported by previous studies where it has been shown that the constitutive exposure of PS on viable cells was not by itself sufficient for efferocytosis by macrophages (Segawa *et al.*, 2011). In fact, reports in the literature suggest that

PS exposure on non-apoptotic cells may serve as a 'tolerate me' signal to stimulate release of anti-inflammatory cytokines (Biermann *et al.*, 2013), this is not something that was investigated as part of the current study. Indeed, it would be interesting to measure cytokine release in this set up to establish if Gal-1 has an anti-inflammatory role via the mechanism of facilitating PS presentation on non-apoptotic neutrophils to macrophages to stimulate their release of anti-inflammatory cytokines.

In summary these findings indicate that exogenous Gal-1 does not modulate the phenotype of neutrophils in a manner that encourages their phagocytic uptake despite the well-established finding that Gal-1 facilitates PS exposure on viable neutrophils. The exact mechanism for this and the outcome remains to be elucidated, however it would suggest an anti-inflammatory action for the protein which may still result in dampening inflammation and thus indirectly reducing inflammatory infiltrate. Importantly, current literature suggests that Gal-1 treatment of macrophages induces a pro-resolving phenotype (Rostoker *et al.*, 2013) and improves their ability to phagocytose (Barrionuevo *et al.*, 2007), therefore illustrating that the role of Gal-1 in efferocytosis is not redundant.

### *4.7. Concluding Remarks*

Protein glycosylation is fundamental to the immune response, with roles in antibody function, antigen presentation and leukocyte recruitment, however it is becoming increasingly obvious that during inflammation the cellular glycome is modified with aberrant glycosylation likely to play a role in numerous pathologies. Galectins have the ability to recognise and bind to sugar molecules and as such their actions are specifically dependent on the expression of distinct saccharides presented on cellular proteins and lipids. It is through binding these molecules that galectins cross-link on the cell surface in order to elicit their down-stream effects. Through further understanding the biology of Gal-1 (as a prototype of immune relevant carbohydrate binding proteins that recognise and bind to specific sugar molecules) anti-inflammatory therapies could capitalise on the sugar code that decorates the majority of inflammatory proteins and lipids modulating their actions.

This research aimed to elucidate the actions of Gal-1 on cell trafficking as well as neutrophil apoptosis and clearance. Under the hypothesis that Gal-1 can reduce both the recurrent movement of white blood cells from the blood into the arthritic joint and the enhanced lifespan afforded to these cells, Gal-1 represents a potential new therapeutic strategy for treating RA. Furthermore, findings from this study will significantly increase the limited existing knowledge regarding the effects of Gal-1 on neutrophils and neutrophil-driven pathologies.

Currently the role of Gal-1 in the resolution process remains to be fully clarified. The delayed remission of arthritis observed here in Gal-1 KO mice coupled with the ability

of Gal-1 to counter-regulate the neutrophil pro-survival signals of pro-inflammatory mediators found in RA synovial fluid is suggestive of a role in neutrophil clearance. Collectively these findings have important implications for the treatment of inflammatory arthritis, where the prolonged survival of neutrophils contributes to the pathogenesis of disease.

## Chapter 5. Future Direction

### *5.1. Model of Acute Inflammation*

Co-administration or prophylactic treatment with hrGal-1 in the zymosan peritonitis model to verify that there is no difference in leukocyte and more specifically neutrophil recruitment in response to hrGal-1 in this model.

### *5.2. Model of Rheumatoid Arthritis*

The lessened disease evidenced in the Gal-1 KO mice compared to WT in the K/BxN serum induced model of arthritis would suggest expansion of the population of effector T cells that have a regulatory/anti-inflammatory phenotype in the absence of Gal-1. Thus, further phenotyping of effector T cells present in the K/BxN serum induced arthritis model to determine the phenotype of the subpopulation driving the increase.

Although findings from this study were not indicative of an effect of the exogenous protein in the K/BxN serum induced model of arthritis, additional assessment is required with adjusted dosing parameters and/or duration of disease, to establish whether hrGal-1 has an effect in this model.

### *5.3. Apoptosis and Phosphatidylserine Exposure.*

For *ex vivo* apoptosis to establish if viable neutrophils taken from Gal-1 KO mice would still progress through apoptosis quicker when plated at equal cell density as WT cells. It would be interesting to investigate this for both inflammatory stimulated and non-stimulated neutrophils.

For *in vitro* apoptosis further studies will look at the intracellular signalling pathways with specific focus on tyrosine phosphorylation of Lyn. Also, to verify that Gal-1 reversal of pro-survival factors occurs through binding to CD45 on the neutrophils surface it would be interesting to block the receptor prior to incubation with hrGal-1 and assess levels of apoptosis.

Regarding increased PS expression seen on neutrophils in response to hrGal-1 it would be pertinent to establish if this was due to Gal-1 crosslinking at the cell surface, thus it would be interesting to look at PS and Gal-1 on the neutrophil surface by microscopy to establish if co-localisation and/or clustering of the molecules is occurring.

Further investigation into the role of AnxA1 release in response to GM-CSF and hrGal-1 is required. It would be interesting to look at granule mobilisation and the interplay of these molecules on this function. Alternatively, a FPR2 antagonist could be used to establish if an AnxA1 feedback loop is being facilitated in this setting.

### *5.4. Macrophages and Efferocytosis*

Full characterisation of Gal-1 null macrophages and WT macrophages in response to exogenous Gal-1 is required in order to build a better picture of the role of Gal-1 on macrophages. Investigating the release of endogenous Gal-1 in response to inflammatory stimuli present in the arthritic joint is still a relatively unexplored aspect of macrophage/Gal-1 biology and would provide further insight on their potential pro-resolving role.

### *5.5. Glycophenotype and Vasculature*

Findings from this study indicate that there may be changes to the vasculature in Gal-1 KO mice compared to WT, thus it would be intriguing to perform permeability assays on Gal-1 KO (potentially endothelial cell specific) and WT mice.

To extend this research, glycophenotyping of immune cells and endothelial cells should be performed to determine modifications in response to inflammation in Gal-1 KO and WT mice.

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